Redirecting interlukin-10 in the fibrotic liver

Mattos Pinto, Adriana

Stellingen behorende bij het proefschrift

Redirecting Interleukin-10 in the fibrotic liver: Effects on the pathogenesis

1. The development of cell-specific targeted cytokines represents a suitable approach to untie the intricate pathophysiology of a disease or it may serve as basis for novel anti-fibrotic therapies. (This thesis)
2. Interleukin-10 delivered to HSC significantly affects macrophage profiles within the liver. (This thesis)
3. The mild and mixed responses seen after administration of native Interleukin-10 is most likely induced by a combination of local effects (leading to enhanced fibrosis) and systemic immunosuppressive effects (leading to reduced fibrogenesis). (This thesis)
4. If you try and take a cat apart to see how it works, the first thing you have on your hands is a non-working cat. Douglas Adams
5. If a cluttered desk is a sign of a cluttered mind, of what, then, is an empty desk a sign? Albert Einstein
6. The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny!". Isaac Asimov
7. The most erroneous stories are those we think we know best – and therefore never scrutinize or question. Stephen Jay Gould
8. I don't know where I'm going, but I'm on my way. Carl Sagan
9. Science should give you wings and freedom and not cages and limitations.
10. Science will be a true treasure when each and every person in the world can equally benefit from it.
11. Não haverá borboletas se a vida não passar por longas e silenciosas metamorfoses. Rubem Alves

Redirecting Interleukin-10 in the fibrotic liver: Effects on the pathogenesis

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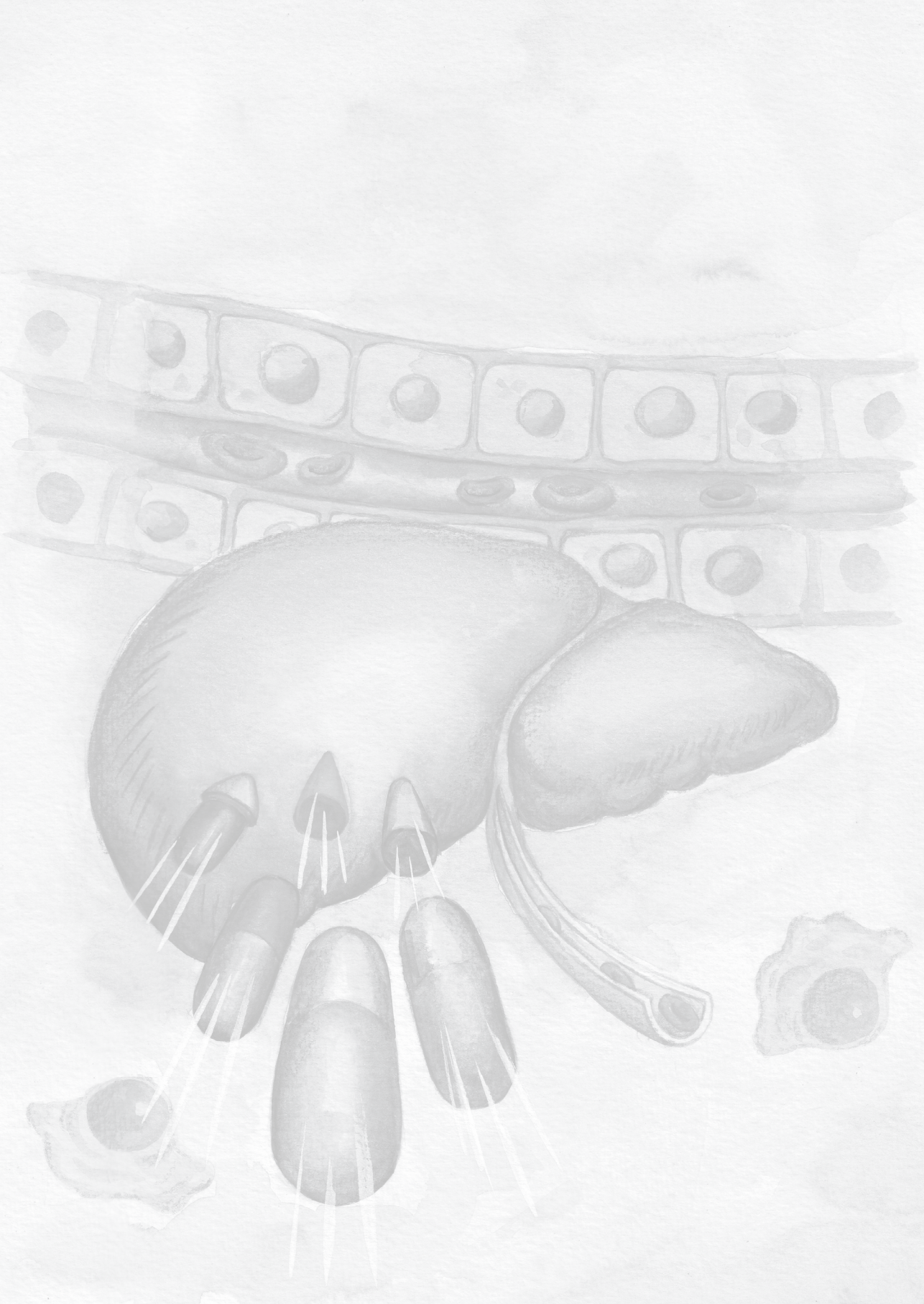

Chapter 1

General introduction, scope and outline of the thesis

"Now, why is the stomach surrounded by the liver?
Is it in order that the liver may warm it and it may in turn warm the food?
This is indeed the very reason why it is closely clasped by the lobes of the liver,
as if by fingers." -- Galen, ca. 200 A.D

The liver in historical context

One of the first representations of liver anatomy and physiology was found in Egyptian papyri, around 1550 B.C. In the 8th century B.C., Homerus described the anatomy of the liver and defined it as a vital organ because he observed that when the liver was wounded it caused death. The study of the liver progressed in antiquity and was subject of great speculations. Ancient Mesopotamian cultures believed that the liver was the center for thought and feeling in the body. The knowledge about the liver reached its highest level with the Greek physician Galien of Pergamon (129-200 A.C), who wrote during the Roman period that the liver was the most important organ in the body because it was the first organ to appear in the formation of a fetus and identified the gall bladder and spleen as the two crucial subsidiary organs of the liver. On the other hand, the macroscopic hepatic structure was defined as "simple" and the liver parenchyma was thought to be derived from the solidification of blood by the body heat.

Leonardo da Vinci (1452-1519) is considered one of the greatest genius of mankind and the father of modern hepatology; he described human liver anatomy and liver cirrhosis. During the 17th century, advances in our understanding of anatomy continued as the English physician Francis Glisson (1597-1677) became the first person in history to write a book devoted exclusively to the liver, the *Anatomia hepatis* (1654) [1]. The Italian physiologist Marcello Malphighi (1628-1694) was the first to use the newly invented microscope to observe the liver. He made a rude description of the hepatic microanatomy and proved that bile was secreted by the liver and not by the gall bladder [2]. In 1833, the Irish surgeon Francis Kiernan (1800-1874) published *The Anatomy and Physiology of the Liver* (4to, 4 plates, London, 1833) and his work is regarded as the first attempt of modern microscopy used in natural sciences [3][4].

Liver structure and function

The liver is the largest internal organ of the human body, weighting between 1.3-3.0 kg and represents about 2% of the total body weight in the average adult human. The liver is essential in keeping the metabolic homeostasis of our body. Among its various functions, we point out the clearance and detoxification of compounds and foreign substances from the blood (metabolism, excretion), synthesis of proteins that participate in the blood clotting and innate immune system, production and excretion of bile fluid, digestion and absorption of fats and fat-soluble vitamins, and storage pool of vitamins, iron and copper etc [5].

The multicellular milieu of the liver is formed by parenchymal cells (the hepatocytes) and non-parenchymal cells, i.e., the sinusoidal endothelial cells (SEC), the hepatic stellate

cells (HSC), and the Kupffer cells (KC). The histological unit of the liver is the hepatic lobule, in which a highly specialized capillary vessel is found: the hepatic sinusoid [6]. The endothelial cell lining is separated from hepatocytes by the space of Disse and is discontinuous, fenestrated, and without a basement membrane [7]. Such morphology facilitates optimal exchange of compounds between hepatocytes and the blood. The normal liver extracellular matrix (ECM), consisting of collagen type IV, fibronectin and laminin, provides a well-balanced scaffold to which cells can adhere and migrate [8].

Hepatocytes are associated with metabolic and secretory liver functions, regulating detoxification of endo- and xenobiotics, production of critical circulating proteins. Death of hepatocytes is correlated with an impairment of the liver function. Upon liver injury, hepatocyte apoptosis is an important trigger in inflammation and fibrosis [9][10].

Kupffer cells are liver tissue resident macrophages, with a pronounced endocytic and phagocytic capacity. Kupffer cells are the largest population of tissue macrophages in the body as they represent 80-90% of the total tissue macrophages [11][12]. These cells are responsible for the clearance of damaged or senescent blood cells [13] and they participate in the innate immune defense as they control early events related to liver inflammation and are specialized in antigen presentation to T lymphocytes [12,14,15]. Macrophages are amongst the most active secretors of mediators of all cells in our bodies, releasing a whole myriad of cytokines, chemokines and growth factors that participate in homeostasis, host defense, inflammation, and fibrosis. Exposure of the liver to damaging agents, such as fat, alcohol, hepatitis B and C virus (HCV and HBV, respectively), bile constituents, may lead to KC activation which results in production of many proinflammatory substances in order to attract other inflammatory cells to the site of injury [16-18].

In a normal liver, from 5-10% of the non-parenchymal cell population is represented by hepatic stellate cells. They are located between hepatocytes and sinusoidal endothelial cells in the subendothelial space [19] and are in intimate contact with the other cells types. This contributes to the intracellular exchange of soluble mediators and cytokines within the tissue [20]. Around 70% of body retinoids (vitamin A and its metabolites) are stored in the liver and the vast majority of it (~95%) can be found in the form of lipid droplets within HSCs [21]. Vitamin A participates in several physiological processes, such as in mammalian reproduction and embryonic development, immune responses, vision, and bone development/growth [22-25]. During liver injury, HSC lose their lipid/vitamin A droplets and become “activated” displaying a myofibroblast-like phenotype. This transformed cell is highly proliferating, fibrogenic and contractile [26]. Activation of HSC is regarded as a key event in the progression of fibrogenesis [27][28][29].

Sinusoidal endothelial cells (SEC) in the liver comprise approximately half of the non-parenchymal hepatic cells. They form unique capillaries with a fenestrated monolayer, without a basement membrane, that separates hepatocytes from the circulating blood [30,31]. SEC are active in hepatic microcirculation, controlling the exchange of

fluids, metabolites, solutes and particles between sinusoidal blood and the liver parenchyma [32,33]. Defenestration of the SEC is a major contributing factor in hepatic dysfunction, as seen in fibrogenesis, and in aging [34,35].

Each cell type in the liver is incredibly diverse in both their biology and interaction with the surroundings (ECM and other cells). A better understanding of these intrincated interactions could lead the identification of targets for an effective treatment against liver fibrosis/cirrhosis.

Liver fibrosis

Advanced fibrosis and cirrhosis represents the main pathophysiological consequence of most chronic liver diseases and this may lead to life-threatening clinical consequences such as liver failure and hepatocellular carcinoma [20,36-38]. In Europe and USA, cirrhosis is the most non-neoplasic cause of death and is the 7th overall cause of death in western countries [39]. Epidemiologic studies predict an increase in the prevalence of the disease in the next two decades largely due to the emergence of hepatitis C virus (HCV), the continued problem of hepatitis B virus (HBV) infection control, and the liver pathologies associated with obesity and chronic alcohol abuse. Because of the reasons listed above, the need for an effective therapy against liver fibrosis is urgent [27,40].

Chronic hepatocyte injury which can be triggered by hepatitis B and C infection, metabolic disorders, alcohol abuse or any other harm to the liver, initiates the development of fibrosis and causes disruption of the fine balanced extracellular matrix (ECM). This results in excess of deposition of fibrotic ECM components in the space of Disse which leads to structural changes in the sinusoidal wall, loss of the EC fenestrations and the development of a fibrotic ECM. At the end of this process, known as capillarization, the structural changes of hepatic sinusoids hampers the metabolic exchange between portal venus flow and hepatocytes, which leads to impaired hepatic functions [41-43]. Throughout fibrogenesis, the composition of liver ECM changes qualitatively and quantitatively. The total amount of components of ECM increases 3-5-fold in hepatic fibrosis and cirrhosis as compared to healthy livers. Interactions between the different liver cells and matrix are important to keep normal liver homeostasis. Disruption of the fine balance of these interactions is a trigger towards hepatic fibrogenesis [44,45][46]. In a healthy liver, the matrix in the space of Disse is composed of low density membrane-like matrix, mainly consisting of collagen type IV, laminin and proteoglycans. In the fibrotic livers the normal ECM is replaced by a matrix rich in fibrillar collagens (types I and III) [47-50], as can be seen in the following scheme (Fig. 1) [43]:

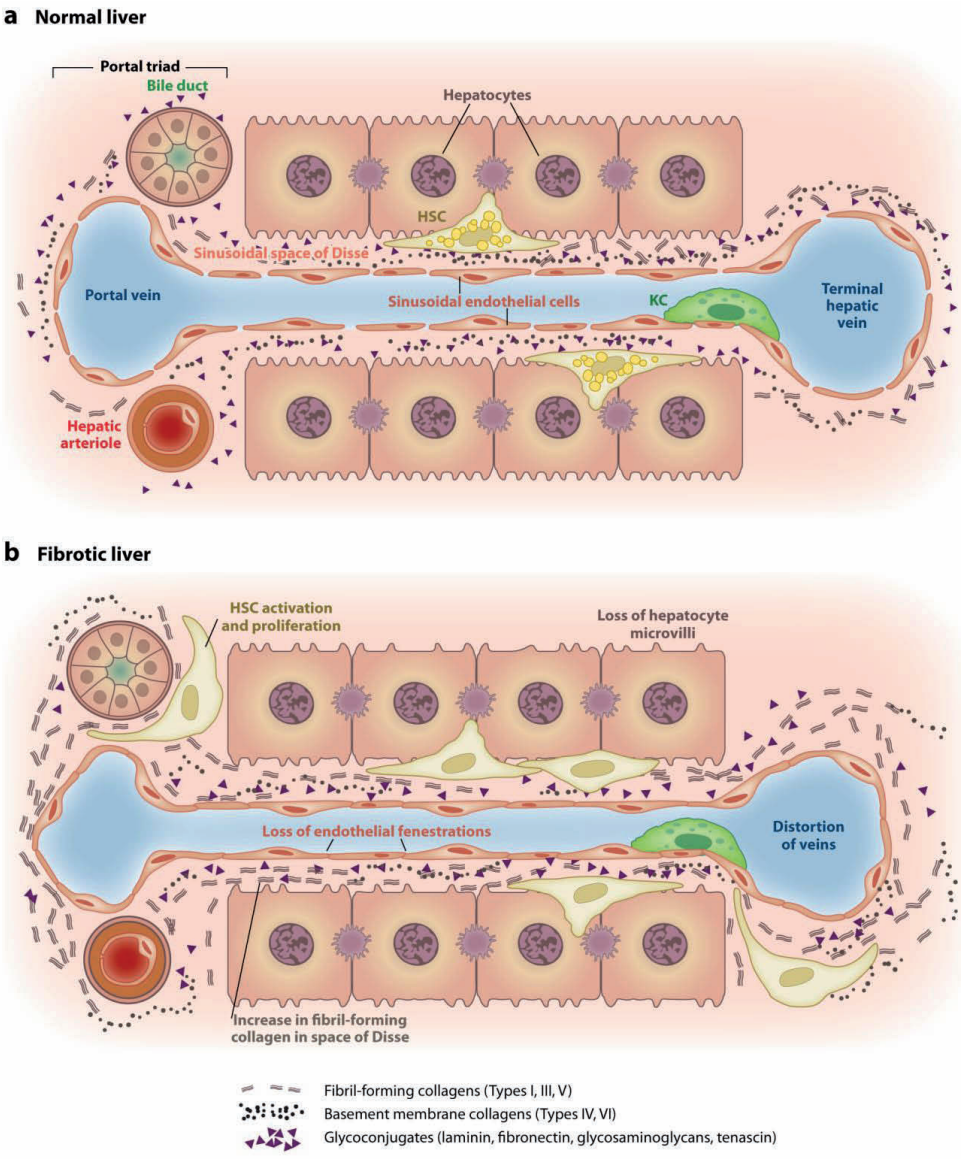

Fig. 1. *Matrix and cellular alterations in hepatic fibrosis. (A) Normal liver parenchyma. Sinusoids are separated from hepatocytes by a low-density basement membrane-like matrix, which ensures metabolic exchange. (B) Deposition of ECM in the space of Disse leads to loss of both endothelial fenestrations and hepatocyte microvilli, which results in impairment of normal metabolic exchange. Permission obtained from Elsevier Ltd © Hernandez-Gea V., Friedman S.L. Annu. Rev. Pathol. Mech. Dis. 2011. 6:425-56*

The fine regulation between fibrogenesis-fibrolysis in the liver ecosystem is due to many factors, but the balance between matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) is crucial in the matrix homeostasis. In rodents, MMP-13 is known to be the major fibrillar collagenase (MMP-1 is the human equivalent) [51] which is mainly secreted by scar-associated macrophages [52]. This MMP is inhibited by TIMPs. Besides the inactivation of MMP-13, TIMP-1 is related to anti-apoptotic effects on activated HSCs and thus contributes even more to fibrogenesis [53].

Hepatic fibrosis and even cirrhosis are known to be reversible if the underlying cause is successfully removed [54][55][56][57] [58][59][60]. Animal studies have shown that during the recovery process of fibrosis degradation of ECM occurred due to decreased levels of TIMP-1 and TIMP-2 and higher collagenase levels [40,61]. Furthermore, resolution of hepatic fibrosis is associated with increased apoptosis of activated hepatic stellate cells and myofibroblasts [61,62][63]. Liver macrophages are now recognized as important players in matrix remodeling. During resolution, scars associated macrophages increase MMP-13 secretion and thus accelerate ECM degradation [64,65].

In the next pages, more details about the two most important liver cell types in fibrotic livers for this thesis are provided: the HSC/ myofibroblast, and the macrophages/Kupffer cells (KC).

Hepatic stellate cells (HSC)/ Myofibroblasts (MF)

In response to hepatic tissue injury, HSCs undergo differentiation from a quiescent cell to an activated myofibroblast. Besides other characteristics, activated HSCs are positive for α-SMA and display high amounts of platelet derived growth receptor beta (PDGFβ-receptor). Furthermore, this activated cell type overproduces fibrillar collagens, particularly types I and III [28,49,66]. Mediators released from damaged hepatocytes and by activated kupffer cells seemed to be the key answer for triggering HSC activation [67,68]. The activation process is driven by cytokines, transforming growth factor-β (TGFβ) and platelet—derived growth factor-BB (PDGF-BB) [20,69].

The understanding that the activation of HSCs is a key factor in liver fibrogenesis has opened new insights in the study of this pathology. Activated HSCs coordinate the whole process of fibrosis, via interaction with other hepatic cells and with immune cells. Activation of the immune response through secretion of chemokines and cytokines, increasing angiogenesis, and regulation of oxidant stress are some of the activities of HSC [70].

Activation of HSCs occurs in two major sequential stages: initiation and perpetuation (fig.2). A resolution phase might follow, if the injury is withdrawn. During the ***initiation phase***, the quiescent HSC becomes responsive to cytokines and growth factors and a rapid upregulation of the PDGFβ-receptor follows while a myofibroblast-like phenotype develops. The ***perpetuation phase*** refers to the ability of activated HSCs to maintain their activated status, via auto and paracrine stimulation, and to secrete excess of ECM components; especially collagens type 1 and III [20][70]. ***The resolution*** of hepatic fibrosis is supported by strong evidences, although there are still doubts about its complete reversibility. A critical mechanism that underlines the reversion of liver fibrosis is HSC apoptosis, senescence or quiescence [71-73].

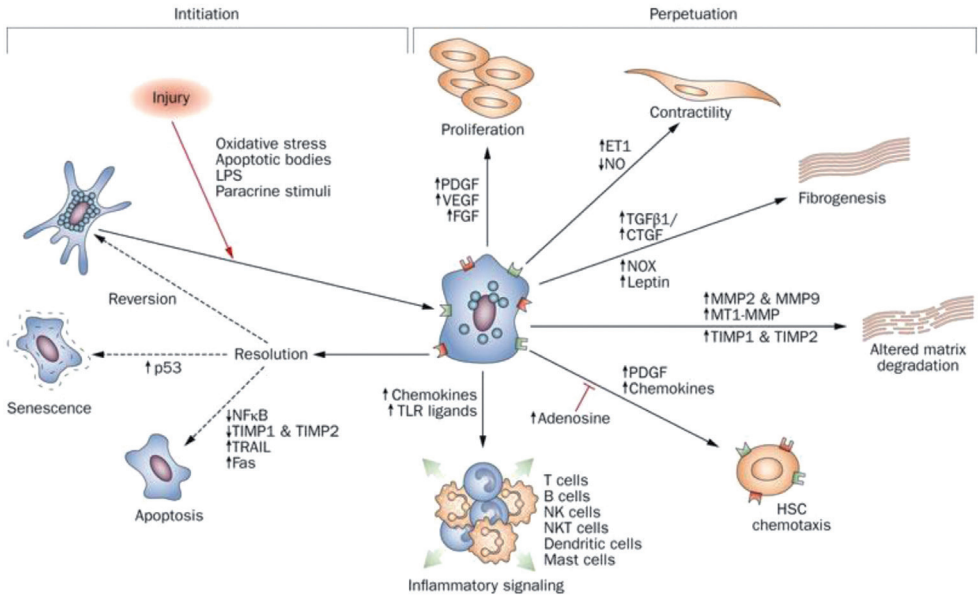

Fig. 2. *HSC activation.* ***Initiation*** *is stimulated by soluble stimuli such as oxidant stress signals (reactive oxygen intermediates), apoptotic bodies, LPS and paracrine stimuli from neighboring cell types including Kupffer cells, sinusoudal endothelium and hepatocytes. Initiation is followed by* ***perpetuation****. Proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis and inflammatory signaling are specific phenotypic changes characteristics of perpetuation.* ***Resolution*** *of hepatic fibrosis, which occurs following clearance of the primary liver disease, leads to loss of activated HSCs, either through apoptosis, senescence or reversion of activated cells to a more quiescent phenotype.*
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During the resolution phase of fibrosis, apoptosis and reversion of HSCs was revealed in a number of experiments *in vivo* [55,61,73,74]. Thus the induction and/or acceleration of apoptosis in HSC has been a strategy largely used to try to find out novel treatments for liver fibrosis/cirrhosis [75-78].

Hepatic myofibroblasts

The source of hepatic myofibroblasts remains unclear and there is a speculative proposal that different types of liver injury might recruit MFs from different sites; in spite of that, there is ample evidence that point out that hepatic stellate cells (HSC) and the portal fibroblasts are the main sources of activated MFs in the fibrogenic liver [79]. The possible origins of the MFs in liver fibrosis are summarized in the schematic illustration below (Figure 3):

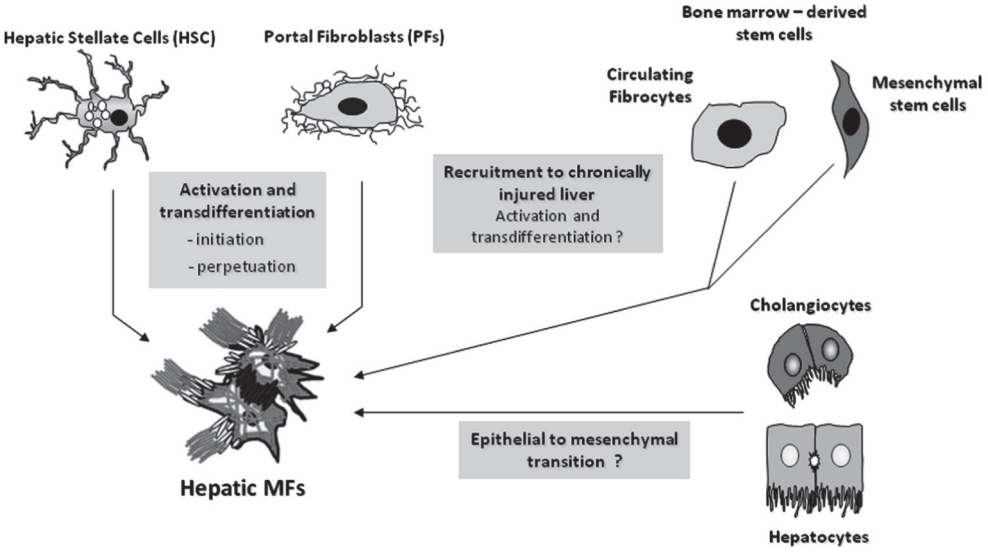

Fig. 3. *Origin of hepatic myofibroblast-like cells.*
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Kupffer cells (KCs)

Until recently, the participation of KC in the pathogenesis of hepatic fibrosis has been mostly related to inflammation and activation of HSC [28]. Upon hepatic injury and hepatocellular necrosis, Kupffer cells are activated and start to express proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6, chemokines, such as monocyte chemotactic protein 1 (CCL2/MCP-1) [80][16] , enzymes, for example the inducible nitric oxide synthase (iNOS) [17], and the growth factors TGFB en PDGF-BB. The release of these substances lead to infiltration of the tissue with additional immune cells and this resulting inflammatory scenario leads KC to also synthesize profibrogenic factors, such as transforming growth factor beta 1 (TGF-β1) which activates HSC and thus initiates over production of ECM [28,81]. Liver macrophages also produce metalloproteases (MMPs) to degrade matrix en thus to reduce fibrosis [56,82,83].

The diversity in biological activities of macrophages supports the evidence that these cells are, in fact, a combination of phenotypically diverse subpopulations [84]. Growing evidence in the past few years indicates that the role of KC and recruited monocytes/macrophages into the fibrotic liver is much more complex than thought before [85-87]. In response to various mediators in the extracellular milieu/environment, macrophages display a complex functional and phenotypic plasticity [84,86,88]. Nowadays, two major activated macrophage subpopulations are proposed: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 and M2 macrophages mediate opposing and complementary functions in tissue inflammation and fibrosis. M1 macrophages are associated with proinflammatory responses and are activated by type I cytokines (eg. Interferon-γ). Besides this contribution of M1 macrophages to tissue injury, these cells also possess anti-fibrotic properties such as production of MMPs that promotes ECM degradation and facilitates resolution of fibrosis. M2 macrophages are supposed to be anti-inflammatory and profibrotic, and are involved in wound repair, producing large amounts of TGFβ and induce myofibroblast proliferation [88-91]. M2 macrophages can be further subdivided into different subtypes, depending on the stimulating factor. M2a macrophages are activated by IL-13 and IL-4, M2b by immune complexes combined with IL1-β or LPS, and M2c are activated by IL-10, TGFβ or glucocorticoids. Alternatively activated macrophages (M2) are balancing M1 activities and thus helping in the maintenance of tissue homeostasis.

Macrophage activation and differentiation into subtypes is a complex and dynamic process in which the same macrophage can transdifferentiate several times and play different roles in the process of inflammation, and fibrosis progression and resolution [65,92-94]. The classification of macrophages into two major polarized

phenotypes could be, however, an oversimplification of the complexity and dynamics of these cells in response to changing microenvironments [84,88,94]. In 2008, Mosser proposed an alternative classification based on the 3 major functions of macrophages: host-defense macrophages, wound-healing (or scar-associated) macrophages, and regulatory macrophages [93]. In addition to these proposed classifications, some articles are revealing new categories of macrophages, as for example a recent publication has shown that activated HSC were also able to trigger the polarization of macrophages *in vitro*. This recently described phenotype comprises M1 and M2 characteristics, being pro-inflammatory (M1) and profibrotic (M2) at the same time and does not fit in any other described category [95].

The importance of Kupffer cells and its phenotypically distinct populations are being recognized as of great importance for fibrogenesis and fibrolysis [65,96-98] and a further characterization of the subpopulations and its roles in fibrosis will add value to research new potential therapeutics for treating fibrotic diseases [95,96].

Interleukin-10 (IL-10) and IL-10 receptor

Interleukin-10 (IL-10) is considered to be the most powerful anti-inflammatory cytokine and was first identified in 1989 as a pleotropic Th2 cytokine that inhibits interferon-gamma (IFNγ) synthesis by T helper 1 (Th1) lymphocytes [99]. IL-10 inhibits cell-mediated immune responses while enhancing humoral immunity [100,101]. Since its discovery, IL-10 has been attracting interest of several study groups which extensively described its structure and biological functions. IL-10 is crucial in limiting the damage in inflammatory and autoimmune diseases due to its anti-inflammatory and immunomodulatory pathways [102,103]. But there is much more about this particular and intriguing cytokine.

The location of the 5.1kb and 4.7kb pairs from both mice and human IL-10 gene respectively, is on chromosome 1. It encodes 5 exons which are separated by four introns. The homology between murine and human IL-10 (hIL-10) is about 80 % [104]. IL-10 gene promoter can have a large number of polymorphisms which are associated with expression of different forms of IL-10 and are these are related to several diseases [105-108], including liver pathologies [109,110]. The mature hIL-10 is a 37-kDa homodimer protein, with two non-covalently bonded identical monomers (with 160 amino acid residues each), forming a V-shape molecule [111][112]. Only the dimer has biological activity and monomerization occurs even under physiological conditions (37°C and low protein concentration) [113].

IL-10 receptor (IL-10R) is a specific cell surface receptor complex and it is composed of 2 different chains, IL-10R1 and IL-10R2 [114,115]. Interleukin-10 dimer

first binds to IL-10R1; IL-10/IL-10R1 interaction will then induce the formation of the binding site for IL-10R2 and the signaling complex is created [105,116,117].

Within the liver, macrophages are known to express IL-10R [101,118,119]. HSC holds a lesser amount of IL-10R and upon activation, the expression levels are up-regulated [119]. To our knowledge, hepatocytes lack IL-10 receptor.

IL-10 in liver fibrosis

Cytokines are soluble protein molecules involved in intra- and inter-cellular communication. They are essential for the embryonic development and the regulation of all cell types; several essential physiologic processes are mediated by cytokines, such as body growth, hematopoiesis, immunity and inflammation [120]. They consist of families and subfamilies that include interferon family, growth factor family, chemokines, tumor necrosis factors and interleukin family. Virtually all cells in the body secrete cytokines, including all liver cell types [121]. Any disruption in the cytokine balance might lead to a wide range of pathologies [122-125]. In most tissues, including the liver, constitutive production of cytokines is very low or even absent; nevertheless, upon physiologic or pathologic stimuli, cytokine production is upregulated and these molecules govern the tissue response to the stimuli then [126]. Within the liver, cytokines are released upon pathological stimuli and they are involved in inflammation, cell necrosis and apoptosis and induction of fibrosis and regeneration following liver injury [127].

The cytokine interleukin-10 (IL-10) is known to have potent anti-inflammatory and antifibrotic properties in the liver [128-131]. IL-10 can downregulate several proinflammatory cytokines in macrophages, including IL-1β and IL-6, and it decreases the expression of MHC class II molecules [132,133]. It is also postulated that IL-10 can prevent activation of quiescent HSC, which is assessed by a downregulation of the number of α-SMA-positive cells, and IL-10 can cause apoptosis of activated HSCs [134,135]. In 2003, a clinical trial with patients with hepatitis B-induced fibrosis revealed a downregulation of inflammatory and fibrotic parameters, but the study was abandoned due to an increased viral burden most probably caused by the immunosuppressant activities of IL-10 [136]. *In vitro* experiments in HSC showed that IL-10 downregulates collagen I and increases expression of matrix metalloproteases and thus promotes degradation of collagens I and III [137-141]. The role of IL-10 in the resolution of liver fibrosis can be related to the up-regulation of matrix metalloproteinase-13 (MMP-13) and the inhibition of tissue inhibitor of metalloproteinase-1 (TIMP-1) [142,143]. MMP-13 is responsible for the degradation of collagen I and III [119,128,137,144] and is mainly secreted by scar-associated macrophages

during the resolution phase of liver fibrosis [82]. TIMP-1 is secreted mainly by activated HSC and is responsible for the inactivation of MMP-13 and the subsequent increased deposition of extracellular matrix (ECM). Another possible explanation for the antifibrotic properties of IL-10 is that its anti-inflammatory effects could result in less fibrogenesis [128,145]. However, anti-inflammatory treatments mostly do not lead to reduced fibrosis [146].

In the other hand, several *in vivo* studies have shown that IL-10 possess profibrotic effects rather than antifibrotic. Mice genetically deficient in IL-10 develop reduced lung fibrosis in response to silica particles [147][148]. As IL-$10^{(-/-)}$ mice displayed a downregulation of TGF-β by alveolar macrophages (AM) and an upregulation of prostaglandin E2 (PGE2), it was hypothesized that the profibrotic activity of IL-10 in lungs could be mediated by the induction of TGF-β expression in lung macrophages and the inhibition of PGE2 secretion by fibroblasts, both induced by IL-10 [149]. Other studies, using a transgenic mice model that is characterized by lung- specific human IL-10-overexpression, showed increased tissue inflammation and fibrosis [150]. This same transgenic mice model was investigated in 2011 and revealed that IL-10 overexpression drives lung fibrosis via the recruitment of fibrocytes which is mediated by the CCL2/CCR2 axis. The profibrotic effects of IL-10 were associated with M2 macrophage polarization in the lungs [151]. Corroborating these data, IL-10 is considered to be a Th2 cytokine which generally leads to profibrotic activities [152,153] and indeed an association between the development of fibrosis led by IL-10 and the polarization of macrophages to an alternative phenotype (M2-like) was noticeable in some studies [149,154-157]. So, IL-10 has important inhibitory functions in inflammation but while many reports describe anti fibrotic effects of IL-10, just as many describe pro-fibrotic effects of this cytokine.

Signaling pathway and biological effects in liver fibrosis

The binding of IL-10 to its receptor activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. Classically, this signaling pathway is related to inflammation and ECM remodeling [158]. Two members of the Janus tyrosine kinase family are involved in IL-10 signaling pathway: JAK1 (R1) and Tyk2 (R2) [159]. The activation of JAK1 and Tyk2 phosphorylates the transcription factor STAT3 (Signal Transducer and Activator of Transcription 3) which translocates to the nucleus and regulates target gene transcription (Moreno, 2008; Kong, 2012). STAT3 activates expression of genes that negatively regulate inflammation and enhances fibrosis such as TGF-β1 [158,160,161]. IL-10/IL-10R interaction and signaling pathway are illustrated in the scheme bellow (Fig. 4) [105]:

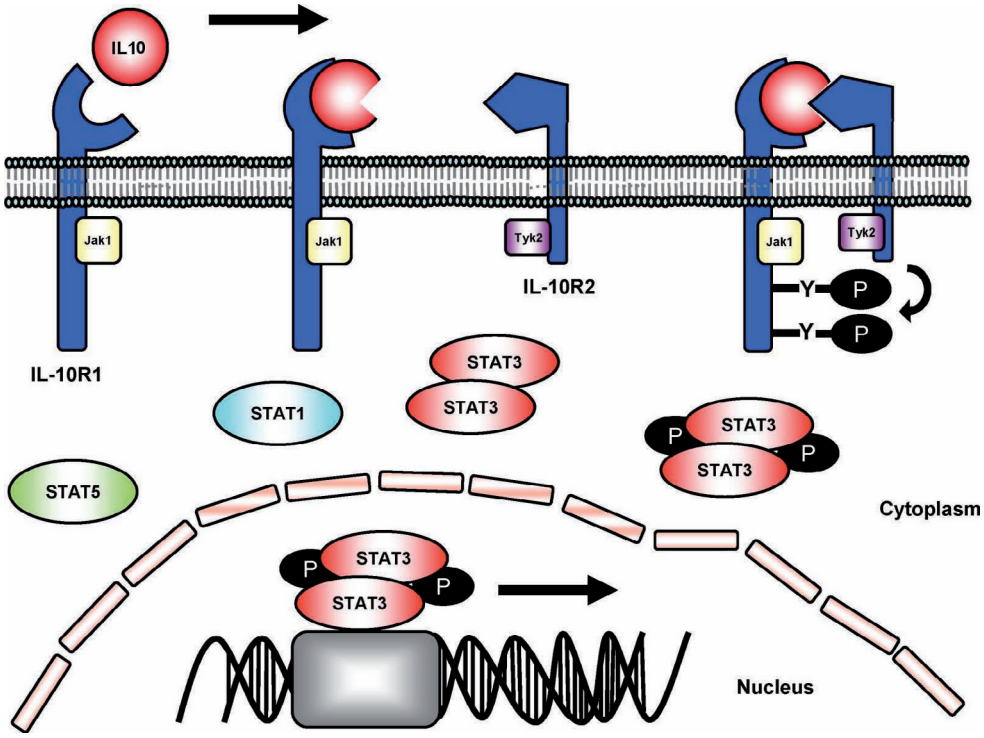

Fig. 4. *IL-10-IL-10R interaction. The cellular IL-10R is a complex composed of the CRF2 members IL-10R1 and IL-10R2. IL-10 first binds IL-10R1. This interaction apparently leads to a comformation change of the cytokine creating a binding site for IL-10R2.*
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In the liver, STAT3 can be activated by several other factors than IL-10 (including IL-6, IL-22, EGF, hepatitis viral proteins) and this transcription factor is known to play a role in acute phase response, protection against liver injury and liver regeneration via induction of STAT3 in hepatocytes [162]. Activation of STAT3 in hepatocytes might constrain liver fibrosis by stimulating hepatocytes to secrete unknown soluble factors that inhibit HSC activation [163]. However, evidence has shown that IL-10 does not significantly induce STAT3 activation in hepatocytes, but suggests it may activate this pathway in non-parenchymal cells and the hepatoprotective effects would be related to the negative regulation of inflammation [164]. On the other hand, STAT3 also enhances hepatic fibrosis in non-parenchymal cells through the upregulation of TGF-beta1 expression. The suppressor of cytokine signaling 3 (SOCS3) might prevent fibrogenesis by inactivating STAT3 [158].

The role of STAT3 in hepatic fibrosis *in vivo* is not known due to the non-existance of a HSC-specific STAT3 knockout mice [165], but deficient leptin or leptin pathway rodents are resistant to the development of liver fibrosis [166][167][168]. Leptin is known to be increased in overweight people and is an adipocyte derived hormone [169] that mediates liver fibrosis thought activation and survival of HSC via STAT3 signaling *in vivo* [170]. STAT3 activation in KC is also an important mechanism to exacerbate liver fibrosis promoting TGF-β1 production [171]. In addition, STAT3 is also involved in the leptin- and IL-6-mediated production of TIMP-1 in HSCs and hepatocytes, respectively [172][173]. TIMP-1 is a survival factor for HSCs; thus, activation of STAT3 in HSCs and hepatocytes may increase liver fibrogenesis via the upregulation of TIMP-1.

However, the activation of STAT3 by IL-10 in Kupffer cells and macrophages may prevent liver inflammation and fibrogenesis because it represents a key anti-inflammatory signal for the attenuation of liver inflammation [174,175][176]. The role of STAT3 in liver fibrosis remains largely unclear. Besides the recent new data on this subject, further studies are necessary to illuminate the specific role of this pathway in each cell type in the liver [165]. Furthermore, the IL-10-specific STAT3 effects in different cell types involved in the hepatic fibrogenic process and its interaction with other cytokines and distinct/overlapping pathways would provide an important lead for future therapies.

Drug targeting approach

Biological compounds, including cytokines, are considered to be the future drugs for the treatment of chronic diseases. Currently some cytokines are approved by The Food and Drug Administration (FDA) for clinical use. For example, IL-2 (Proleukin®) is used for the treatment of metastatic renal cell carcinoma and metastatic melanoma, and Interferon-α2b (PEGIntron A®) for Kaposi's sarcoma, chronic hepatitis B infections, chronic hepatitis C infections, follicular (non-Hodgkin's) lymphoma, and chronic myelogenous leukemia.

The therapeutic use of cytokines in case of fibrosis may be beneficial because these compounds are very potent (picomolar to nanomolar range) and they play essential roles in the pathogenesis of this disease. However, several problems can be encountered in the therapeutic application of cytokines [177][178]. One major problem is the short plasma half-life (often minutes) of cytokines due to efficient degradation by enzymes and rapid renal clearance. Another problem is that if the body concentration of the cytokine is increased with higher and/or more frequent doses, the occurrence of (undesirable) side effects is increased due to the expression of their receptors in various cells within the body. These two main complications that jeopardize cytokine therapy might be solved by the use of drug targeting techniques in which the cytokine is selectively delivered to a target cell type [179][180][181]. The challenge in cytokine therapy is to

improve the pharmacokinetic profile of cytokines and to target the cytokine to a cell of interest while maintaining the biological activity even after chemical modification.

During fibrosis/cirrhosis the uptake of drugs by various liver cells can be reduced because of excess of ECM, shunting, and impaired liver functions can interfere with the drug uptake, metabolism and distribution among the hepatic cells [181]. Hepatic stellate cell proliferation and activation is a key event in hepatic fibrogenesis and thus this cell is a perfect target to treat this pathology [49]. Our group has successfully delivered several drugs and biologicals to activated HSC and observed downregulation of essential fibrotic parameters [75,182][180]. During pathogenesis, diseased cells show a different receptor profiling, where some receptors are downregulated while other displayed an upregulation. Cell-specific delivery of cytokines as performed in our group takes this as an advantage, with targeting of the desirable therapeutic drug to a receptor that is preferably expressed by the (diseased) target cell. This novel strategy aims to improve the pharmacokinetics and efficacy while at the same time avoids unwanted side effects. This goal can be fulfilled by chemical modification of the cytokine with homing devices that recognize certain receptors present on the target cell [75,183-186].

Besides being a promising novel approach to treat chronic diseases, a myriad of potential uses exist for cell-specific drug targeting. This technology will enable researchers to examine the consequences of targeting several biologicals to diverse target cells both in pathologic and healthily conditions. Taken liver fibrosis as an example, the effect of different cytokines in each hepatic cell type and the resulting responses and interactions during fibrogenesis could open the door for better understanding of the intricate relationship between cells and fibrogenic mediators as well as unraveling the pathways involved in the pathophysiology of the disease. Because of the reasons explained above, this technology might lead to novel treatments for liver fibrosis and several other pathologies.

In my thesis, IL-10 is studied for its applicability as a therapeutic cytokine for the treatment of liver fibrosis as wells as for unraveling IL-10-specific effects on HSC *in vivo during* fibrogenesis. This will be described in more detail, in the scope and aim of this thesis.

Scope and aim of this thesis

The studies described in this thesis focus on the role of interleukin-10 (IL-10) during hepatic fibrosis, specifically on its effects on hepatic stellate cells (HSC) *in vivo* after its delivery to this cell-type.

As outlined in the general introduction, IL-10 plays an important role in the regulation of both the progression and resolution of fibrosis in several organs. Hepatic fibrosis is a very complex disorder with the intricate participation of extracellular matrix components, several hepatic cells and a variety of endogenous substances. The

elucidation of these interactions and pathways is essential to design new drugs for anti-fibrotic treatment. We used a cell-specific delivery approach to examine the effects of IL-10. The complex activities of this cytokine on different cell types, comprising both anti- and pro-fibrotic effects, are outlined in the introduction. Cell-specific targeting of cytokines may represent a novel anti fibrotic treatment but it may also provide a tool to unravel the specific effects of a particular cytokine on a particular hepatic cell type. We aimed to use a cell-specific delivery approach to unravel the effects of IL-10 on hepatic stellate cells during CCl_4-induced liver fibrosis in mice.

In order to evaluate the best option for specific delivery of compounds to HSC, we first evaluated in **chapter 2** [75] two possibilities for selective targeting of a biological compound (in this case 15-deoxy-Δ12, 14-prostaglandin J2) to the HSC. Both synthesized carriers consisted of human serum albumin (HSA) modified with either mannose-6-phosphate (M6P-HSA) or with PDGF-receptor recognizing peptides (PPB-HSA). M6P-HSA binds to the M6P/Insulin-like Growth Factor II receptor, whereas PPB-HSA binds to the Platelet Derived Growth factor Receptor (PDGF-R). Both receptors are abundantly expressed on activated HSC. The binding and biological effects of both conjugates were evaluated in primary isolated rat HSC and we also studied the pharmacokinetics of the conjugates by comparing the *in vivo* organ distribution of 15dPGJ2-M6PHSA and 15dPGJ2-PPBHSA with HSA. Cellular specificity of both conjugates within the diseased livers was determined by intrahepatic immunohistochemistry. In addition to that, the presence of the target receptors for these conjugates, i.e. the M6P-IGFII – and the PDGF-receptor, was determined in normal and cirrhotic human liver tissues.

As the plasma half-life of IL-10 is very short [187] we modified IL-10 with polyethylene glycol (PEG) with the purpose of prolonging its circulation time and enhancing its effects. PEGylation of small proteins is a well-known strategy to achieve this and it has led to several new therapeutic drugs against liver diseases, i.e. Pegasys and Pegintron against hepatitis B and C. PEGylation of IL-10 has however not been examined yet. **Chapter 3** shows studies on the chemical modification of IL-10 with either 5 or 20 kDa PEG and the two compounds were compared in relation to circulation time, liver accumulation and effects on decreasing in fibrotic parameters. An increased degree of PEGylation of cytokines leads to an increased circulation time in plasma but this is often paralleled by an attenuated interaction with its receptor, and thus to an attenuated effectivity. In the studies presented in Chapter 3 we aimed to explore this balance by using two different PEG chains of different lengths.

We then aimed to specifically target IL-10 to the HSC during hepatic fibrogenesis. For this purpose we chemically engineered IL-10 with PPB, using 2kDa PEG as linker (IL10-PEG-PPB), as described in **chapter 4**. The PPB-peptide ensures delivery of IL-10 to the PDGFβ-Receptor which is abundantly expressed on HSC. The

conjugate was characterized and tested *in vitro* for biologic activity. *In vivo* effects were explored in mice with CCl_4-induced chronic fibrogenesis. We examined the effects of the conjugate, as compared to free IL-10 and untreated animals, on collagen type I, MMP-13, TIMP-1 and macrophage profiling. To further elucidate the role of IL-10 in the cellular communication between HSC and liver macrophages during fibrogenesis *in vivo* and to unravel the mechanism by which selective targeting of IL-10 to HSC affected fibrogenesis, we further explored the effects of PPB-PEG-IL-10 in the same mice model (**chapter 5**). In this study, other parameters, such as collagen type III, fibronectin, TGFβ and the CCR2/CCL2 axis were analyzed. Additional data on the accumulation of different macrophage phenotypes were also obtained. Our data show increased fibrogenesis after delivery of IL-10 to HSC. These pro-fibrotic effects appear to be mediated by the influx of macrophages of the M2c subtype that express a pro-fibrotic phenotype. Our studies illustrate the interaction between HSC and liver macrophages and the role of IL-10 in this.

This is the first study demonstrating that specific targeting of IL-10 to HSC *in vivo* leads to effects on the development of hepatic fibrosis associated with macrophage polarization. Since hepatic fibrosis is a very complex pathological process and up to now there is no treatment available for fibrosis in any organ, the development of cell-specific targeted cytokines or other biological drugs could mean a suitable approach to untie the intricate pathophysiology of the disease or it may serve as basis for novel anti-fibrotic therapies.

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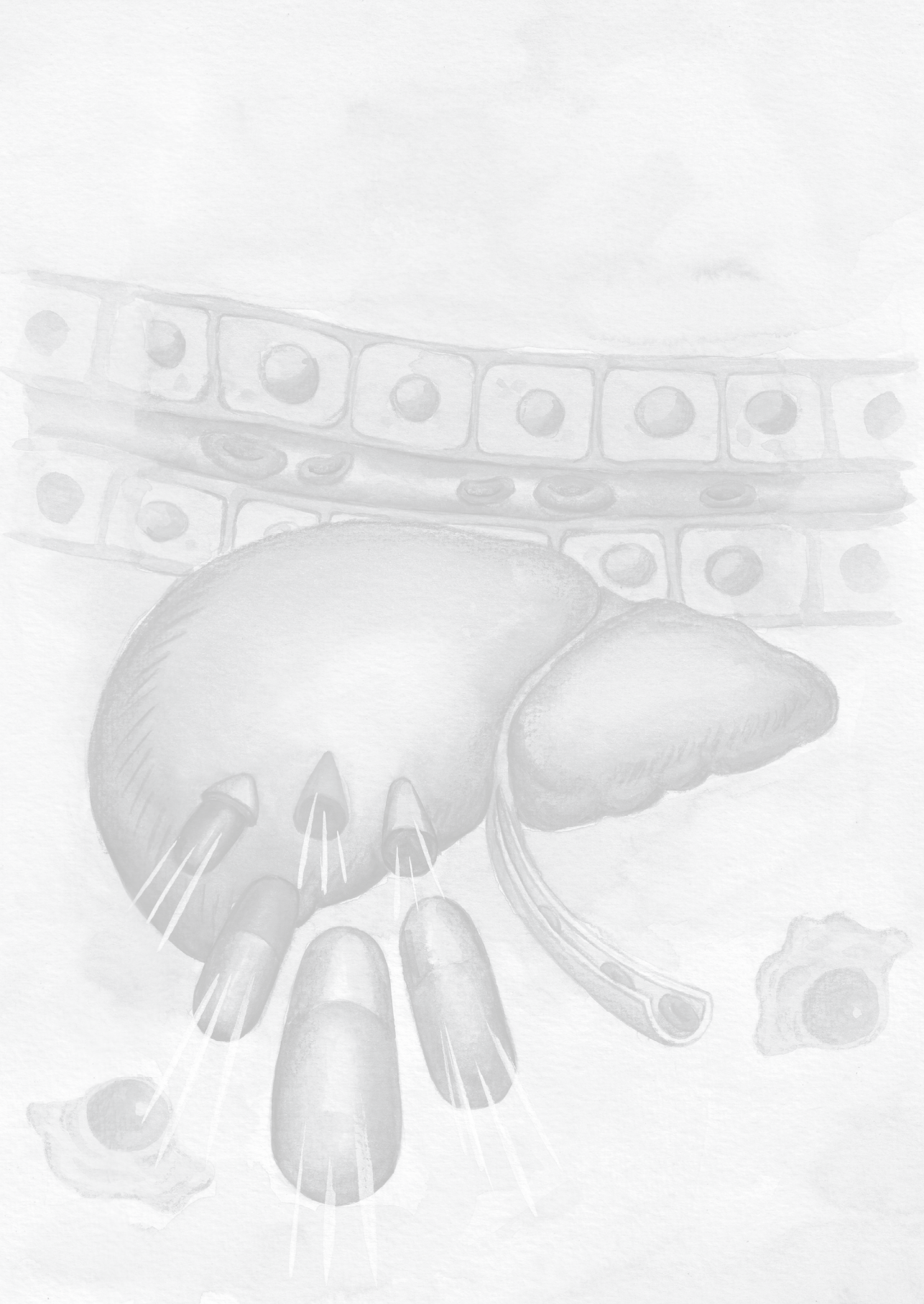

Chapter 2

Targeting 15d-Prostaglandin J2 to Hepatic Stellate Cells: Two Options Evaluated

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Abstract

Purpose. Delivery of apoptosis-inducing compounds to hepatic stellate cells (HSC) may be an effective strategy to reverse liver fibrosis. The aim of this study was therefore to examine the selective targeting of the apoptosis-inducing drug 15-deoxy-D12, 14-prostaglandin J2 (15dPGJ2) with two different HSC carriers: human serum albumin modified with the sugar mannose-6-phosphate (M6PHSA) or albumin modified with PDGF-receptor recognizing peptides (pPBHSA).

Methods and Results. After chemical conjugation of 15dPGJ2 to the carriers, the constructs displayed pharmacological activity and specific receptor-mediated binding to HSC in vitro. Unlike 15dPGJ2- pPBHSA, the cellular binding of 15dPGJ2-M6PHSA was reduced by a scavenger receptor antagonist. In vivo, both conjugates rapidly accumulated in fibrotic livers. Intrahepatic analysis revealed that 15dPGJ2- M6PHSA mainly accumulated in HSC, and to a lesser extent in Kupffer cells. 15dPGJ2-pPBHSA also predominantly accumulated in HSC with additional uptake in hepatocytes. Assessment of target receptors in human cirrhotic livers revealed that M6P/IGFII- receptor expression was present in fibrotic areas. PDGF-b receptor expression was abundantly expressed on human fibroblasts.

Conclusions. These studies show that 15dPGJ2 coupled to either M6PHSA or pPBHSA is specifically taken up by HSC and is highly effective within these cells. Both carriers differ with respect to receptor specificity, leading to differences in intrahepatic distribution. Nevertheless, both carriers can be used to deliver the apoptosis-inducing drug 15dPGJ2 to HSC *in vivo*.

Key words: *antifibrotic drugs, drug targeting, hepatic stellate cells, liver fibrosis, 15dPGJ$_2$*

Introduction

Liver fibrosis may occur as a response to chronic liver injury and may lead to cirrhosis. HSC play a pivotal role in this fibrogenic process, because this cell starts to proliferate and accumulate within the injured liver. During this activation process, increased amounts of collagen and other extracellular matrix compounds and inhibitors with matrix degrading activity are produced by these HSC, thereby strongly affecting the liver architecture and eventually liver function [1][2]. Up till now, no pharmaceutical intervention is available to treat this fibrotic disease [3]. The application of antifibrotic drugs has not been successful, partly because these drugs do not accumulate in the target cells in the diseased liver or cause side effects elsewhere in the body. Alteration of the pharmacokinetic characteristics of the drug by means of drug targeting may represent a promising approach in the development of an effective antifibrotic drug.

In recent years, different HSC-selective carrier systems have become available [4-6]. One system uses the sugar mannose-6-phosphate which is coupled to the protein human serum albumin to form $M6P_{28}HSA$. This carrier binds to mannose-6-phosphate/insulin-like growth factor-II (M6P/IGFII) receptors [4][7-10]. The expression of these M6P/IGFII receptors increases as a result of HSC activation during liver fibrosis [11] enabling selective accumulation of M6PHSA in HSC of fibrotic rats [4].

In other delivery systems for HSC, the homing devices are cyclic receptor-recognizing peptides [5,6]. The carrier homing to the PDGF-β receptor (pPBHSA) may be quite relevant, since PDGF-BB is the major cytokine involved in the proliferation of HSC during the fibrotic process, and the PDGF-β receptor is highly upregulated on activated HSC [12,13]. The carrier pPBHSA competitively inhibited the binding of PDGF-BB to its receptor, and in vivo preferentially homed to the HSC in fibrotic rat livers with minor uptake in hepatocytes [5]. In vivo, uptake was associated with disease-induced expression of the PDGF receptor in liver and kidney.

In experimental liver fibrosis, resolution of the disease was found to be accompanied by loss of HSC via spontaneous induction of apoptosis in these cells [14,15]. Therefore, induction of apoptosis in HSC might represent an attractive approach to reverse this liver disease. In this respect, $15dPGJ_2$ is interesting, because it was shown that this prostaglandin induced apoptosis of human hepatic myofibroblasts [16]. In addition, sub-apoptotic concentrations of $15dPGJ_2$ significantly inhibited the expression of interstitial collagens as well as its proliferation in human hepatic myofibroblasts *in vitro* [17,18]. $15dPGJ_2$ was also found in vivo at sites of inflammation during the resolution phase of the disease, suggesting that it might function as a negative feedback regulator of the inflammatory process [19].

However, generally the pharmacokinetic properties of prostaglandins limit their therapeutic use. Prostaglandins are locally acting mediators that are rapidly metabolized

by the lung [20] or in plasma [21] and excreted from the body via the kidneys resulting in a very short half life [22]. For example, prostaglandin E_2 (dinoprostone) has a plasma half-life ($t_{1/2}$) of less than 1 min and prostaglandin I_2 (epoprostenol) has a $t_{1/2}$ of 2–3 min [23]. The $t_{1/2}$ of 15dPGJ2 is not known. In addition, the high protein binding of prostaglandins in serum [24] might prevent $15dPGJ_2$ to effectively reach the essential cells within the liver after systemic administration. In line with this, in vitro studies demonstrated that pharmacological effects of $15dPGJ_2$ were abolished in the presence of serum [16]. Specific delivery of prostaglandins to HSC might overcome these problems.

The aim of this study was therefore to chemically couple $15dPGJ_2$ to two different HSC-selective drug carriers, i.e., M6PHSA and pPBHSA, and to compare their effectiveness by studying the uptake and pharmacological effects within the target cells.

Material and methods

Materials

HSA was purchased from the Central Laboratory of Blood Transfusion Services (Sanquin Blood Supplies, Amsterdam, The Netherlands). $15dPGJ_2$ was purchased from ITK Diagnostics (Uithoorn, The Netherlands). All other chemicals used were of analytical grade.

Animals

Wistar rats (male, outbred strain, obtained from Harlan, Horst, The Netherlands) were housed under standard laboratory conditions and had free access to food and water. This study was performed in accordance with ethical regulations imposed by Dutch legislation.

Synthesis

M6PHSA

HSA was modified with mannose-6-phosphate groups as described [4]. The monomeric fraction was separated from the dimeric and polymeric fraction by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Uppsala, Sweden) in an FPLC system. The monomeric protein was characterized for protein, sugar, and phosphate content.

pPBHSA

The cyclic peptide C*SRNLIDC* was prepared by Ansynth Service BV (Roosendaal, The Netherlands) and covalently coupled to HSA as described [5].

Conjugation of 15dPGJ2 to the Albumin Carriers

The carboxylic acid group of 15dPGJ$_2$ (10 mg; 32 µmol) dissolved in 150 µl dimethylformamid (DMF)) was activated with 12.5 mg (61 µmol) N,N-dicyclohexylcarbodiimide (DCC, Sigma, St. Louis, MO, USA), in a ratio 15dPGJ2:DCC = 1:2, dissolved in 80 µl DMF and stirred for 1 h (RT). This 15dPGJ$_2$/DCC solution was subsequently added to 20 mg (0.25 µmol) M6PHSA or pPBHSA (dissolved in PBS, 4 mg/ml) in ratio 15dPGJ2: carrier = 130:1, and the mixture was stirred at RT during 16 h. The obtained solutions were extensively dialyzed (Slide-A-Lyzer 10K, Pierce Biotechnology, Rockford, IL, USA) against milliQ water at 4°C for 2 days followed by overnight dialysis against 5 mg/ml HSA solution to remove all low molecular weight compounds and unbound 15dPGJ$_2$. The conjugates were lyophilized and stored at −20°C. The amount of 15dPGJ$_2$ coupled to the carrier was assessed using the 15dPGJ$_2$ correlate-EIA kit (Assay Designs, Ann Arbor, Michigan, USA).

Rat Hepatic Stellate Cell Isolation

We isolated primary HSC from livers of rats (±500 g body weight) using in situ perfusion and subsequent digestion of the livers with collagenase P, DNAse (both Roche Diagnostics, Indianapolis, IN, USA) and pronase E (Merck, Darmstadt, Germany). After separation of the HSC from other hepatic cells by density-gradient centrifugation, the HSC were collected on top of a 12% Nycodenz-solution [25]. Subsequently, the cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), and incubated in a 5% CO_2 humidified atmosphere at 37°C. Cells cultured for 10 days were used for further experiments. At this time point, all cells have an activated phenotype. This was routinely microscopically evaluated and regularly checked by staining for α-smooth muscle actin using a monoclonal antibody (clone 1A4, Sigma).

Binding to HSC

To assess the cellular binding of the conjugates as described [7], 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA were radioactively labelled with ^{125}I [26].

One hundred thousand cells (10 days after isolation) were grown in a six well-plate and pre-incubated with 1% bovine serum albumin in DMEM to block non-specific binding. HSC were then incubated at 37°C with 100,000 cpm of ^{125}I-labelled conjugate in DMEM with 0.2% bovine serum albumin. In addition, wells were co-incubated with HSA, M6PHSA, pPBHSA (all 1 mg/ml) or polyinosinic acid (PIA, 10 µg/ml). After 2 h, cells were washed with PBS and the cell-associated radioactivity was measured on a γ-counter (Riastar, Packard instruments, Palo Alto, USA). These experiments were performed with cells of three separate HSC isolations.

Biological Effects on HSC

HSC (day 7 after isolation; 5,000/well) were incubated in a 96-well plate for 24 h in 200 µl medium with serum, then washed with serum-free medium and incubated for an additional 24 h in 200 µl medium with or without 10% FCS. Subsequently, $15dPGJ_2$, $15dPGJ_2$-M6PHSA, M6PHSA, $15dPGJ_2$-pPBHSA or pPBHSA were added in increasing concentrations and the cells were incubated with or without 10% FCS for another 18 h. Alamar blue (20 µl, Serotec, Oxford, UK) was added and the cells were incubated for an additional 24 h (i.e., final analysis of HSC at day 10 after isolation). The conversion of Alamar blue by the metabolic activity of the cells reflects the number of cells (i.e., net result of proliferation and apoptosis) present in each well. The Alamar blue conversion was measured with a fluorimeter [27]. These experiments were performed with cells of three separate HSC isolations.

Animal Model of Liver Fibrosis

To induce liver fibrosis, rats (220–240 g) were subjected to bile duct ligation (BDL) under isoflurane/O_2/N_2O anesthesia. The rats were used for further experiments at 10 days after ligation; when HSC are activated and excess of extracellular matrix deposits are clearly present (portal expansion with occasional portal-to-portal bridging).

Organ Distribution

BDL rats were injected intravenously, in the penile vein, with a tracer dose of ^{125}I-HSA, ^{125}I–$15dPGJ_2$-M6PHSA or ^{125}I–$15dPGJ_2$-pPBHSA (1.10^6 cpm per rat, n = 3/group) under anaesthesia. Fifteen minutes after injection, the animals were sacrificed, blood was obtained by heart puncture and organs were removed. The radioactivity in each organ was measured with a γ-counter (Packard instruments). The total radioactivity measured in the total organ was corrected for the blood-derived radioactivity according to standard procedures [6].

Hepatic Localization

Anaesthetized BDL rats were injected intravenously with 2 mg/kg $15dPGJ_2$-M6PHSA or $15dPGJ_2$-pPBHSA (n = 3/group). Fifteen minutes after administration, the rats were sacrificed and sections of the liver were frozen in isopentane (–80°C).

Immunohistochemical analysis of acetone-fixed cryostat sections (4 µm) of the livers was used to localize the carrier. Therefore, livers were double-stained with a polyclonal antibody against HSA (MP Biomedicals, Zoetermeer, The Netherlands)

combined with an antibody specific for either the Kupffer cells (ED2, Serotec), sinusoidal endothelial cells (RECA1, Serotec) or HSC (Desmin and Glial Fibrillary Acid Protein (GFAP) simultaneously, both Sigma) [4]. The double-stained sections were quantified by two independent observers (at least ten microscopical fields at 20 × 10 magnification). The total number of double positive cells (red staining reflects HSA-positive cells and blue staining reflects the cell marker) was counted and divided by the total number of HSA-positive cells (all red staining) in the same microscopical area. This yielded the relative uptake of the conjugate by each cell-type.

Localisation of Target Receptors in Human Tissue

Acetone-fixed cryostat sections (4 μm) of human livers from cirrhotic patients (n = 3) and healthy human livers, obtained from donor livers (n = 3), were stained according to standard immunohistochemical techniques [28]. The primary antibodies used in this study were polyclonal antibodies directed against the M6P/IGFII-receptor (K-21) and PDGF-β receptor (958) (both Santa Cruz Biotechnology, CA, USA). After incubation with two secondary antibodies, amino-ethyl carbazole (AEC) was used to visualize the staining.

The scientific use of human liver tissue was approved by the Medical Ethical Committee of the University Medical Center Groningen (The Netherlands).

Statistics

Results are expressed as ±SEM. Statistical analysis was performed by two tailed Student's t-test. P values lower than 0.05 were considered statistically significant.

Results

$15dPGJ_2$ was chemically linked via its COOH-group to the free NH2-group of the lysines as present in M6PHSA and pPBHSA. Using ELISA techniques to detect $15dPGJ_2$, we assessed the presence of $15dPGJ_2$ in the lyophilized and purified conjugates, while the carriers alone did not give an ELISA-signal. We calculated that, respectively, six and eight molecules $15dPGJ_2$ were coupled to M6PHSA and pPBHSA. Subsequently, we confirmed that after conjugation of the carriers with the lipophilic drug $15dPGJ_2$, the ability of both conjugates to bind rat HSC was preserved. Radioactive studies showed significant binding of both constructs to HSC *in vitro* (Fig. 1). To confirm that the cellular internaltization of $15dPGJ_2$ conjugated with M6PHSA or pPBHSA occurred via two different receptors, i.e., respectively, M6P/IGFII receptor and PDGFβ-receptor, we co-incubated the radioactive labeled conjugates with excess amounts of

unlabeled M6PHSA or pPBHSA. For these studies, we used tracer amounts of the radioactive compounds, because tracer doses are representative for all non-saturated dosages. The negatively charged molecule polyinosinic acid, a well-known antagonist of scavenger receptors [29], was included in these studies because coupling of approximately 33 M6P groups to albumin significantly increases the net negative charge of the protein, which implicates that the scavenger receptor might be involved in the uptake of M6PHSA by HSC.

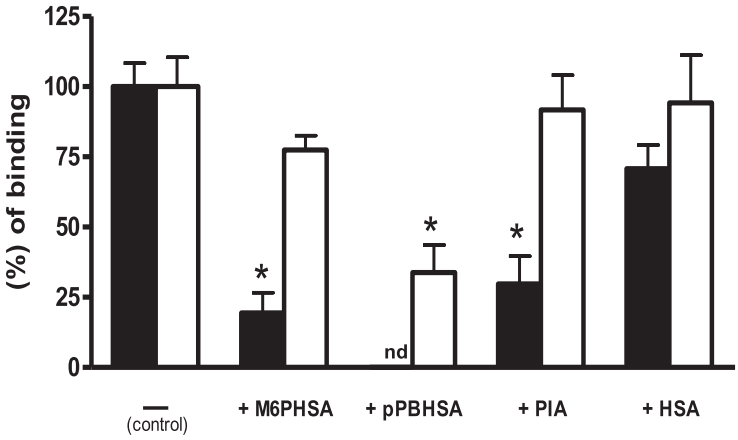

Fig. 1. *Binding of the conjugates ^{125}I–15dPGJ2-M6PHSA (black bars) and ^{125}I–15dPGJ2-pPBHSA (white bars) to cultures of activated rat HSC. Incubation of ^{125}I-labelled conjugate and excess amounts of unlabeled M6PHSA, pPBHSA, the scavenger receptor antagonist polyinosinic acid (PIA), and HSA was performed to assess receptor-specific uptake of the conjugates by the HSC. The results of separate experiments with three different HSC isolations are expressed as the mean ± SEM (* = P < 0.05 as compared to control bar). nd = not determined.*

In Fig. 1 it can be seen that binding of ^{125}I–15dPGJ$_2$-M6PHSA to HSC was significantly reduced by 81 ± 5% ($P < 0.05$) after co-incubation with an excess of M6PHSA, a ligand for the M6P/IGFII receptor. Excess of HSA alone did not significantly reduce the binding of ^{125}I–15dPGJ$_2$-M6PHSA to the HSC. Co-incubation with polyinosinic acid resulted in a reduction of cellular binding by 70 ± 10% ($P < 0.05$). This indicates that the binding of 15dPGJ$_2$-M6PHSA to target cells is mediated by M6P/IGFII receptor and scavenger receptors present on the cell membrane.

The receptor-mediated binding of the other carrier, ^{125}I–15dPGJ$_2$-pPBHSA, was significantly reduced with an excess of pPBHSA. This protein reduced the binding of 15dPGJ$_2$-pPBHSA to HSC by 66 ± 10% ($P < 0.05$). Neither co-incubation with HSA nor M6PHSA or polyinosinic acid reduced the binding of ^{125}I–15dPGJ$_2$-pPBHSA to the HSC, indicating the specificity of its binding to the cell membrane. These studies show that the binding and uptake of 15dPGJ$_2$ coupled to the carrier M6PHSA occurs via different cell membrane receptors than 15dPGJ$_2$ coupled to pPBHSA.

Induction of Apoptosis

We subsequently examined the apoptosis-inducing effect of 15dPGJ$_2$ in its free form and conjugated to M6PHSA or pPBHSA by measuring the viability of primary isolated rat HSC. We also incorporated the influence of serum, since serum components such as albumin are known factors that destroy the bioactivity of prostaglandins [24]. This aspect is of course quite relevant in vivo. Addition of native 15dPGJ$_2$ to cells grown without FCS caused a significant and dose-dependent decline in viability (80% reduction at 20 μM 15dPGJ$_2$). Co-incubation of 15dPGJ$_2$ with 10% FCS completely abolished the activity of 15dPGJ$_2$ in cell cultures (Fig. 2a). Subsequently, we examined the effects of both conjugates. Without FSC, both conjugates induced a dose-dependent reduction in the viability of HSC starting at a concentration of 25 μg/ml 15dPGJ$_2$-M6PHSA (corresponding with 1.9 μM 15dPGJ$_2$) and 10 μg/ml 15dPGJ$_2$-pPBHSA (corresponding with 1.0 μM 15dPGJ$_2$). It may be derived from Fig. 2a and b that 15dPGJ$_2$ targeted with pPBHSA is somewhat more potent than 15dPGJ$_2$ targeted with M6PHSA since lower concentrations of conjugated drug result in higher efficacy. However, additional studies are needed to prove this. In the presence of 10% FCS, both conjugates were still able to reduce the HSC viability, although the dose–response curve was somewhat shifted to the right (Fig. 2b, 69% reduction at 250 μg/ml 15dPGJ$_2$-M6PHSA; Fig. 2c, 81% reduction at 250 μg/ml 15dPGJ$_2$-pPBHSA). This small shift is most likely due to a serum effect on cells, i.e., in the presence of serum these cells are more resistant to apoptotic stimuli. When the carriers alone (250 μg/ml of M6PHSA or pPBHSA) were incubated with the HSC cultures no influence on cell viability in both serum and serum-free conditions were measurable (Fig. 2b and c).

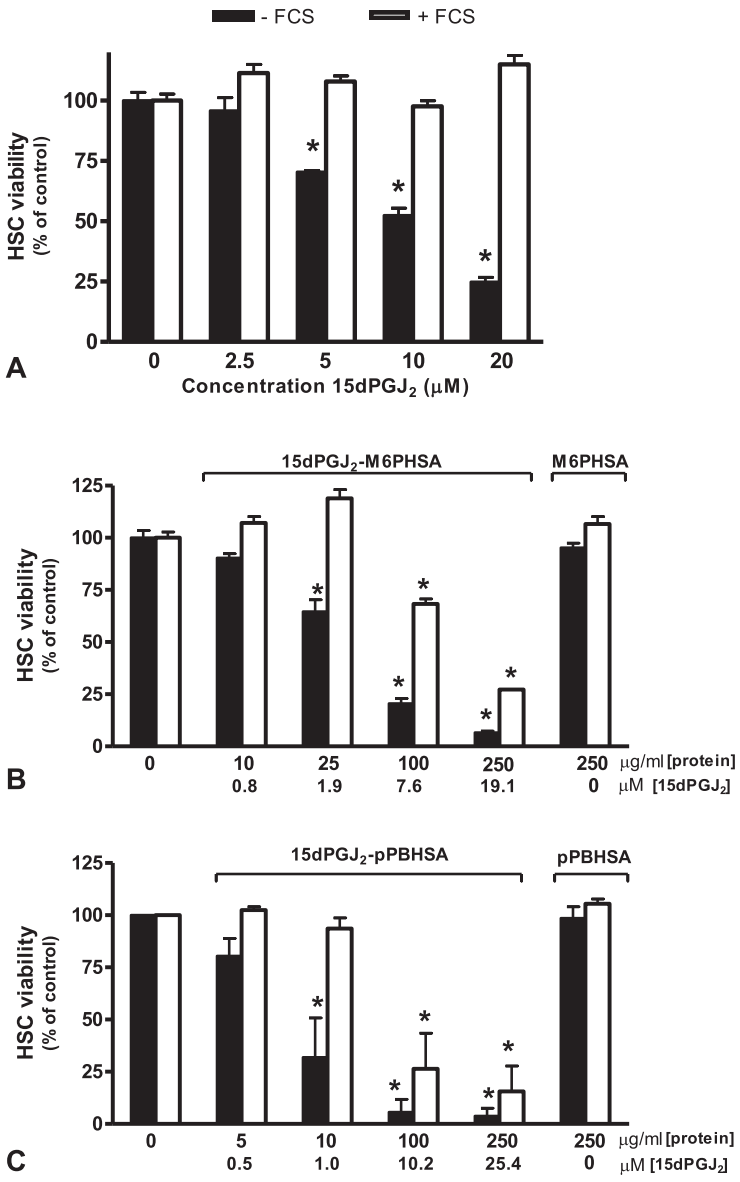

*Fig. 2. Effect of different 15dPGJ$_2$ forms on the viability of isolated activated HSC. (a) 15dPGJ$_2$, (b) 15dPGJ$_2$-M6PHSA and the carrier M6PHSA control, (c) 15dPGJ$_2$-pPBHSA and the carrier pPBHSA control. The experiments were performed under serum starvation (black bars) or in the presence of 10% FCS (white bars). Of both conjugates, the estimated concentration of 15dPGJ$_2$ present in 15dPGJ$_2$-M6PHSA (6 molecules drug/protein) or 15dPGJ$_2$-pPBHSA (8 molecules drug/protein) is depicted as well as their protein concentrations. The control cells (incubated with vehicle) are set at 100% viability and control wells without cells are set as 0% viability. The results are expressed as the mean ± SEM ($n = 3$, * = $P < 0.05$).*

Body Distribution in Rats with Liver Fibrosis

The organ distribution of HSA, 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA was studied in BDL rats, 10 days after induction of the disease (Fig. 3). Fifteen minutes after intravenous administration, both ^{125}I–15dPGJ$_2$-M6PHSA and ^{125}I–15dPGJ$_2$-pPBHSA accumulated significantly in the livers (86 and 63% of the dose, respectively), leaving little conjugate in the blood (3 and 16%, respectively). Less than 3% of the administered ^{125}I–15dPGJ$_2$-M6PHSA and only 7% of the ^{125}I–15dPGJ$_2$-pPBHSA dose was taken up in other organs and tissues. In contrast, organ distribution studies of ^{125}I–HSA in BDL-10d rats revealed that the majority of the injected dose was present in the blood at 15 min after injection (Fig. 3). In conclusion, these data indicate a rapid and almost complete distribution of both conjugates to the fibrotic liver.

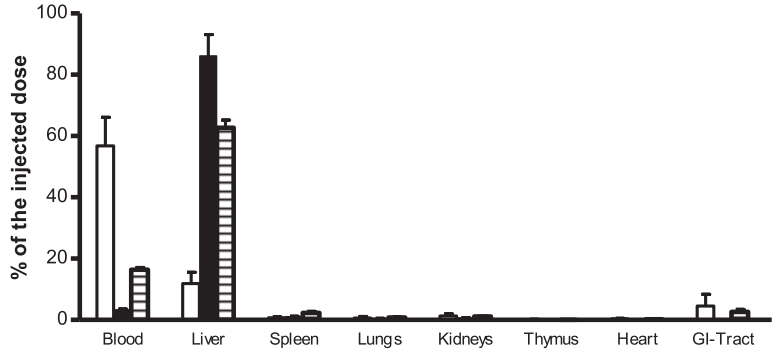

Fig. 3. *In vivo organ distribution of ^{125}I-labeled 15dPGJ$_2$-M6PHSA (black bars), 15dPGJ$_2$-pPBHSA (striped bars), and HSA (white bars) at 15 min after intravenous injection of the compounds in rats with BDL-induced liver fibrosis.*

To determine the cellular specificity of 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA within the diseased livers, we examined the intrahepatic distribution of the conjugates in BDL-10d rats with immunohistochemical techniques (Fig. 4). 15dPGJ$_2$-M6PHSA was clearly taken up by non-parenchymal cells and co-localization with HSC markers was found (Fig. 4a). Quantitative evaluation by counting double-positive cells showed that 64% of the double-positive cells could be identified as HSC (Table I). The second cell type that displayed substantial uptake of the M6P-conjugate was the Kupffer cell (39%). Quantitative evaluation of the cellular distribution of 15dPGJ$_2$ targeted with the other carrier pPBHSA revealed that 57% of the double-positive non-parenchymal cells were identified as HSC (Table I). For this carrier, the hepatocytes also contributed to the uptake of 15dPGJ$_2$-pPBHSA in the fibrotic liver, but our scoring procedure did not allow good quantification of this diffuse staining.

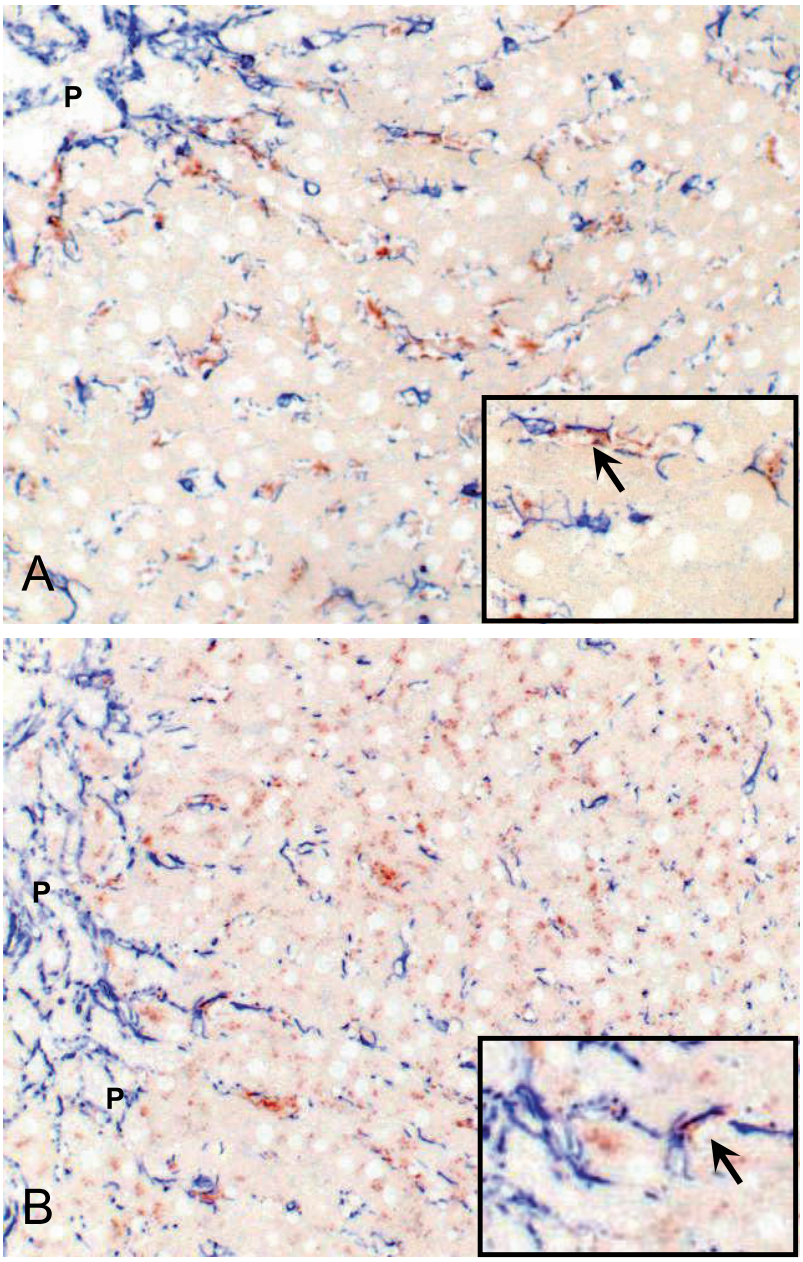

Fig. 4. *Immunohistochemical localization of 15dPGJ$_2$-M6PHSA (a) and 15dPGJ$_2$-pPBHSA (b) in liver of BDL-10d rats, 15 min after intravenous injection of the constructs. Cell-specific localization was determined by double staining of the livers with an antibody directed against HSA (red staining) together with cellular markers to detect HSC (desmin and GFAP; blue staining). Magnification 200×. The inserts shows co-localization of the modified HSA together with HSC (arrow; magnification 400×). P = portal area.*

Table I *Estimation of the Intrahepatic Distribution of 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA in BDL-10d Fibrotic Rat Livers 15 min after Intravenous Injection*

	15dPGJ$_2$-M6PHSA	**15dPGJ$_2$-pPBHSA**
Hepatic Stellate Cells (%)	64 ± 5	57 ± 7
Kupffer Cells (%)	39 ± 3	24 ± 8
Sinusoidal Endothelial Cells (%)	7 ± 5	32 ± 7
Hepatocytes	–	+

The contribution of each hepatic cell type in the total liver uptake of the conjugate was determined by counting the number of double-positive cells (HSA+ and cell marker+) relative to the total number of conjugate-positive cells in the same microscopical field. Values are expressed as the percentage double-positive cells ± SD.

M6P/IGFII and PDGF-β Receptor Staining in Normal and Cirrhotic Human Livers

Finally, we examined the expression of the target receptors for our HSC-specific carriers in histologically normal and cirrhotic human livers (Fig. 5). Normal liver sections displayed a parenchymal staining for the M6P/IGFII receptor. In cirrhotic human livers, M6P/IGFII-receptor expression was still present in hepatocytes. However, additional staining for the receptor was found in cells in the fibrous bands, which are the areas within the liver characterized by excessive collagen deposition. The staining was predominantly detectable on cholangiocytes, both in cells lining the bile duct and bile ductular structures (Fig. 5c).

PDGF-β receptor expression in normal human livers was only found in the portal areas, in which the hepatic artery and portal vein were stained (Fig. 5d). Sinusoidal endothelial cells were not stained for the PDGF-receptor. The expression of the PDGF-β receptor was massively enhanced in cirrhotic human livers (Fig. 5e), and the receptor staining was mainly detectable in cells within the fibrous bands (Fig. 5f). Our observations are in line with the reported enhanced expression of PDGF-β receptor in activated myofibroblasts which are located in these areas [13].

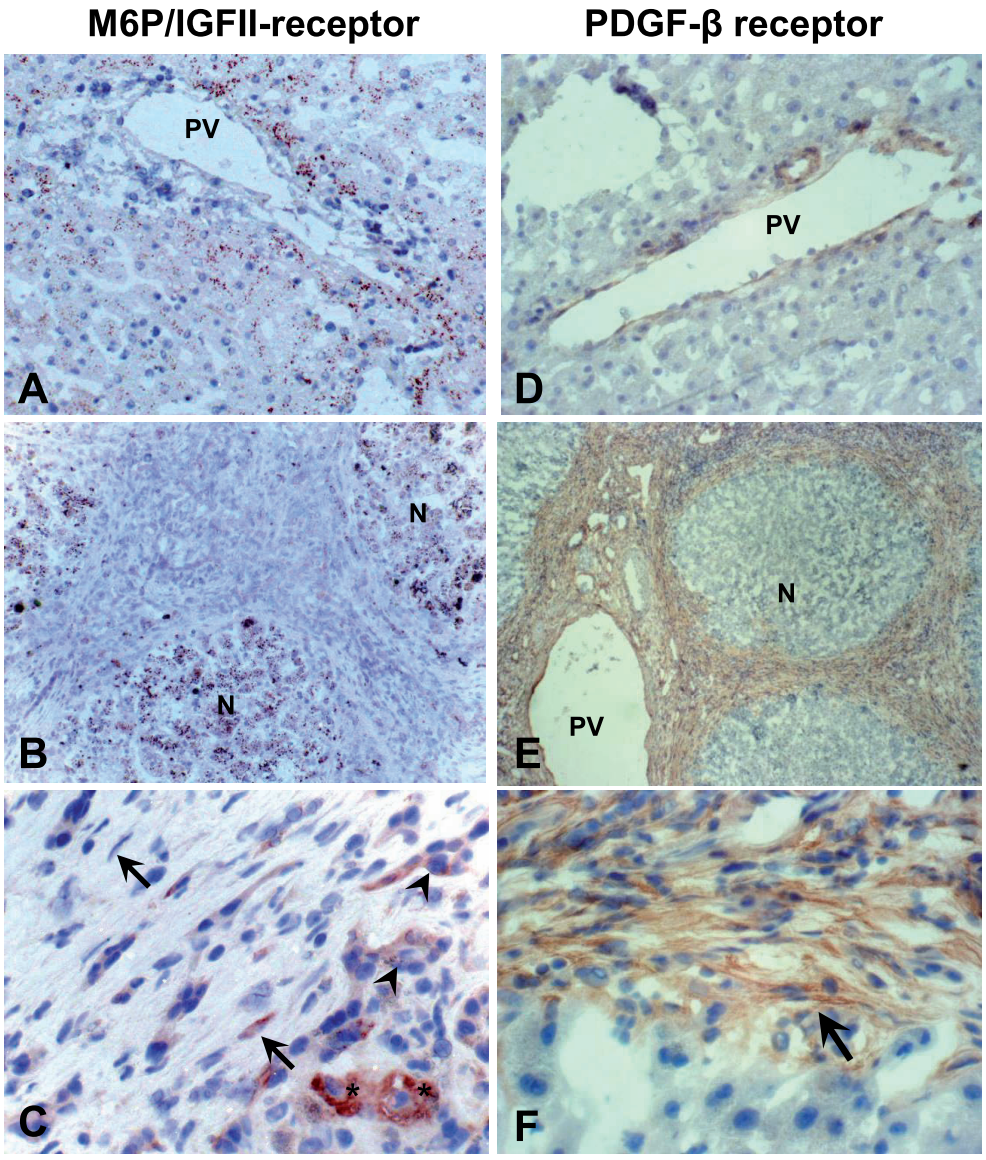

Fig. 5. *Immunohistochemical detection of M6P/IGFII-receptor (a, b and c) and PDGF-β receptor (d, e and f) in healthy human livers (a, d; magnification 20 × 10) and cirrhotic human livers (b, magnification 10 × 10, C magnification 4 × 10). A high magnification (c, f; magnification 400×) shows that cells in the fibrous bands are positive for both receptors. An asterix indicates the localization of periseptal hepatocytes, the arrow head shows ductular cholangiocytes, and the arrow points to (myo) fibroblasts. PV = portal vein, N = cirrhotic nodule.*

Discussion

In this study, we compared two HSC-specific carries for their ability to deliver $15dPGJ_2$ to fibrogenic cells in the liver. The first carrier M6PHSA, which is substituted with sugar groups as homing devices, binds to the M6P/IGFII receptor present on activated HSC [4]. The second carrier pPBHSA, which is substituted with cyclic peptide homing devices, can bind to the PDGF-â receptor which is strongly increased on the cell-surface of activated HSC [5].

The interaction with HSC as reported for the individual carriers [5,7] was preserved after coupling of $15dPGJ_2$ to these carriers. The receptor-mediated binding of both 15dPGJ2 conjugates was inhibited by co-incubation with its own carrier, while no effect of HSA was found. These blocking experiments indicate that binding to these cells is mediated by specific receptors. In addition, co-incubation with polyinosinic acid, a known blocker for the scavenger receptor class A [29], strongly reduced the binding of $15dPGJ_2$-M6PHSA to the cells. The involvement of scavenger receptors was anticipated because these receptors bind negatively charged molecules and M6P-containing compounds have a net negative charge, as confirmed with FPLC-monoQ analysis. The expression of a scavenger receptor class B, CD36, was reported on HSC and a co-localisation with alpha-smooth muscle actin-positive HSC in human fibrotic livers was immunohistochemically demonstrated [30]. However, inhibition by polyinosinic acid, as found in our studies, suggests that not scavenger receptor class B, but rather class A contributed to the accumulation of M6PHSA conjugates in the HSC. This scavenger receptor class A has not yet been described on HSC, but our results together with the observations presented by Adrian et al. [31] warrant further exploration of these receptors on HSC in rat and human livers. In contrast, binding of $15dPGJ_2$-pPBHSA to cultured HSC was not affected by polyinosinic acid, suggesting that scavenger receptors are not involved in the binding of pPBHSA to HSC. Moreover, preincubation with M6PHSA showed that $15dPGJ_2$-pPBHSA was not interacting with any of the binding domains of the M6P-carrier. This shows that both carries use different entry routes to deliver a drug within the HSC.

$15dPGJ_2$ was conjugated to the carrier via its carboxylic acid group (COOH). In contrast to the cyclopentenone ring of prostaglandins, the carboxylic acid group is not essential for the biological activity [32,33]. In our studies, we showed that both conjugates were able to effectively reduce the viability of HSC in vitro. This implicates receptor-mediated endocytosis of the drug-carrier and intracellular degradation of the constructs in the lysosomes of the cell. Of note, the chemical amide bond between the carboxylic acid group of the prostaglandin and the lysine moieties within HSA cannot be cleaved by intracellular proteases as present in the lysosomes with part of the carrier attached to it. Using the same coupling procedures to couple naproxen to

HSA, it was found that naproxen-lysine was released within the cell, after degradation of naproxen-HSA [34]. Also, coupling of mycophenolic acid to HSA via this coupling procedure yielded not the parent compound [35]. So, most likely $15dPGJ_2$ is also not released as the parent compound but despite this pharmacological activity is present.

Because of its apoptosis-inducing activities, $15dPGJ_2$ seems an attractive compound to apply in liver fibrosis [16-18]. However, the therapeutic use of $15dPGJ_2$ may be limited because of high albumin binding [24] and expected poor pharmacokinetics as most of these lipophilic prostaglandins easily cross cell membranes. Also, our studies with HSC in vitro showed that serum completely blocked the activities of free $15dPGJ_2$, while both conjugated forms of $15dPGJ_2$ were still effective and reduced HSC viability almost comparable to serum-free conditions. The pharmacokinetic parameters of 15dPGJ2 after i.v. injection have not been reported, probably because so far no radioactive compound is available and HPLC detection is not sensitive enough. The obstacles as encountered in the therapeutic use of prostaglandins in vivo may be solved by targeting of the prostaglandin to the HSC by means of conjugation to HSC-specific carriers.

With regard to an expected low HSC accumulation of $15dPGJ_2$ in vivo, we now demonstrate a rather complete targeting to the fibrotic liver of both $15dPGJ_2$ conjugated forms. Respectively, 86 and 63% of the dose of $15dPGJ_2$-M6PHSA and $15dPGJ_2$-pPBHSA was present in the liver already 15 min after intravenous injection. Previously, we reported an hepatic accumulation of M6PHSA and pPBHSA of 59 and 48% of the administered dose, 10 min after iv injection [4,5]. The higher liver accumulation as found in the present studies for $15dPGJ_2$ conjugated M6PHSA and pPBHSA may be caused by the attachment of the lipophilic compound prostaglandin. We also determined the intrahepatic localization of both HSC-targeted forms of $15dPGJ_2$. $15dPGJ_2$-M6PHSA predominantly accumulated in the HSC (64%) and Kupffer cells (39%). The high accumulation of the M6P-conjugate in Kupffer cells is probably related to the net negatively charged protein backbone of the construct which is recognized by scavenger receptors (both class A and B) on these Kupffer cells [29]. The other construct $15dPGJ_2$-pPBHSA predominantly co-localizes with HSC and parenchymal cells. Hepatocyte uptake was relatively low, reflected by the diffuse staining and could therefore not be quantified. It is likely that the pPB-conjugate, as also proposed for the carrier pPBHSA [5], follows a similar distribution pattern as the growth factor PDGF-BB, which is taken up by hepatocytes after opsonization by α_2-macroglobulin [36].

Further studies to assess the effects of $15dPGJ_2$ in cells that take up the construct are required. Next to the uptake in HSC, hepatocytes will take up pPBHSA-$15dPGJ_2$ whereas in case of the M6PHSA carrier, Kupffer cells and endothelial cells take up the product parallel to HSC. If effects are found within these cells, it will determine

the choice of carrier. To date, no effects of $15dPGJ_2$ have been reported in KC, EC or hepatocytes. To assess the effects in all cell types and the net effect on liver fibrosis, the constructs have to be administered in therapeutic amounts for a prolonged period of time. This is part of our future research program.

However, the situation within the human diseased liver has also to be taken into account and we decided to focus on this issue first and studied the expression of both receptors in human cirrhotic livers. We demonstrated expression of the M6P/IGFII receptors in hepatocytes of healthy livers, but in human cirrhotic livers an increased expression on fibrogenic cells was observed compared to normal livers. Expression of this receptor on fibroblasts in humans has been reported [37], however no data on hepatic stellate cells or myofibroblasts exist yet. We did find uptake of M6PHSA constructs in human myofibroblasts and liver slices *in vitro* [4], but no significant staining for M6P/IGFII receptor was found in these cells in the studied human livers. Maybe a detailed investigation of M6P/IGFII receptor expression during fibrogenesis in the human liver at various stages of disease and with various etiologies should be performed. The expression of M6P/IGFII receptors in hepatocytes as demonstrated in human livers was also noted in rats [38], but this hepatocyte receptor expression did not lead to any uptake of M6PHSA in this cell type [4,35,39]. Only uptake of M6PHSA in non-parenchymal cells was found, probably the receptor expression in hepatocytes is mostly intracellularly, i.e., not within reach of extracellular plasma proteins.

The highly induced expression of the PDGF-β receptor in human cirrhotic livers as detected by us, correlated with its localization as reported by Pinzani et al. [13]. The high expression of in particular the PDGF-β receptor compared to the expression of the M6P/IGFII receptor makes our carrier with the PDGF receptor-recognizing peptides, pPBHSA, the most attractive candidate for application in the human situation. Subsequent studies to examine the distribution of the drug carriers in human livers or patients will be necessary to examine the applicability of the carriers in the human situation, but at least both target receptors are present.

In summary, the coupling of $15dPGJ_2$ to the HSC-specific drug carriers M6PHSA and pPBHSA clearly led to a rapid and near complete accumulation of the prostaglandin in the fibrotic liver with significant uptake in HSC. Both targeted forms of $15dPGJ_2$ were pharmacologically effective *in vitro* and coupling evidently improved the activity of this prostaglandin in the presence of serum. In vitro receptor interactions differed between both targeted constructs, which led to differences in the hepatocellular distribution in vivo. Studies on long-term effects and on the applicability in patients will be necessary to determine which construct is the most effective during disease. Nevertheless, in both cases, the HSC is the main target cell and in view of the apoptosis-inducing activities of this prostaglandin in HSC, these conjugates may be a valuable tool to attenuate the fibrogenic process within the liver.

Acknowledgments

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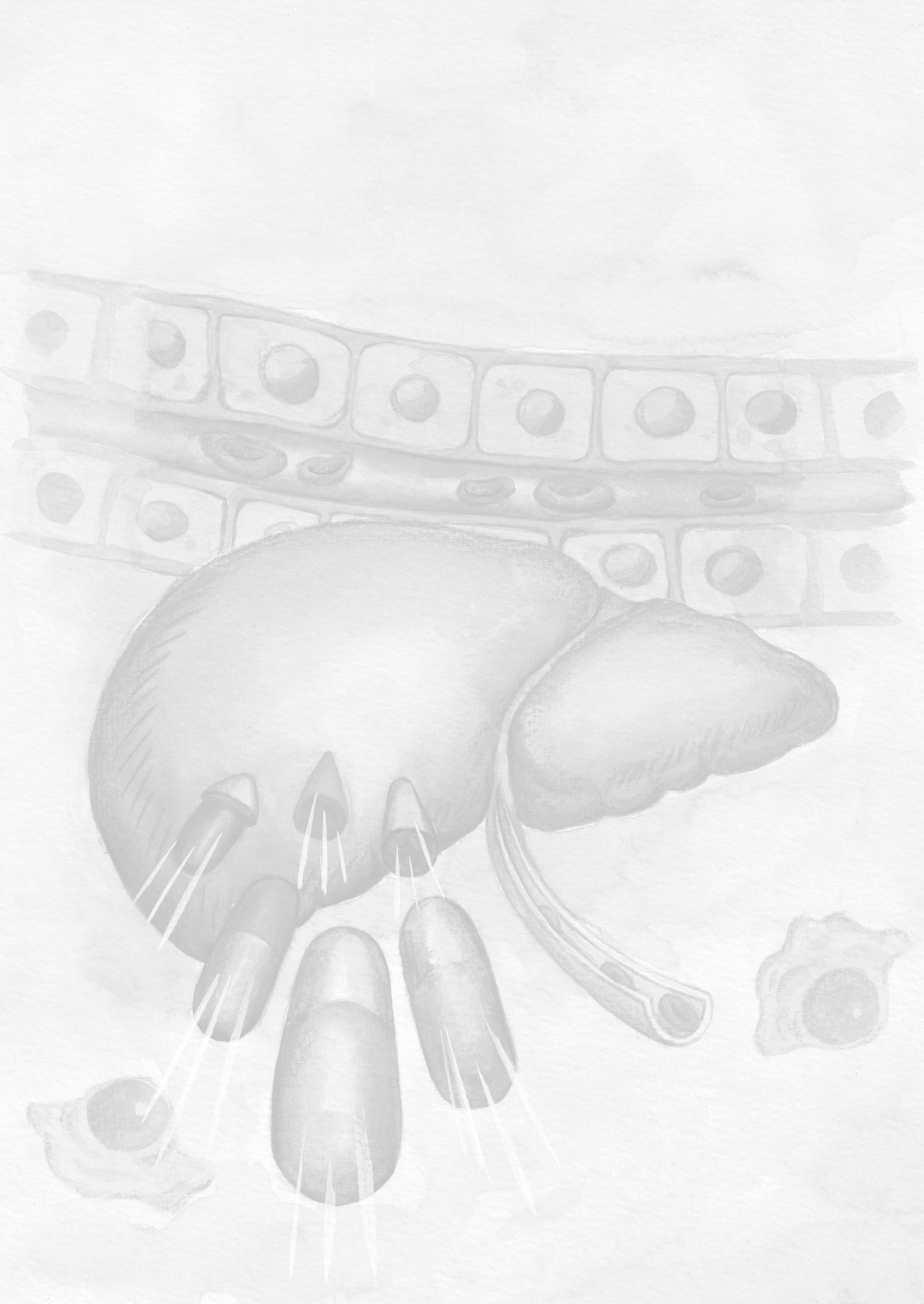

Chapter 3

PEGylation of Interleukin-10 improves the pharmacokinetic profile and enhances the antifibrotic effectivity in CCl4-induced fibrogenesis in mice

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Abstract

Liver fibrosis represents a scar formation process as response to chronic injury and a major cause of death worldwide. To date, no drug is available for this condition. Interleukin-10 (IL-10) has potent anti-inflammatory and antifibrotic properties but its short half-life in the circulation hampers its clinical use. Our aim was therefore to modify IL-10 with polyethyleneglycol (PEG) to prolong its circulation time and enhance its effectivity. IL-10 was modified with 5 or 20 kDa PEG. The biological activity was preserved after PEGylation as assessed by inhibition of TNF-α production by macrophages. *In vivo,* during CCL_4-induced fibrogenesis in mice, both 5PEG-IL-10 and 20PEG-IL-10 showed a longer circulation time compared to IL-10, which was associated with a significant increased liver accumulation. Immunohistochemical analysis of fibrotic livers of mice receiving treatment with IL-10 or its PEGylated forms, revealed a decrease in markers reflecting HSC and KC activation induced by 5PEG-IL10. Transcription levels of IL-6 were decreased upon treatment with IL-10 and both PEGylated forms, whereas IL-1β levels were only down-regulated by 5PEGIL-10 and 20PEGIL-10. We conclude that PEGylation of IL-10 is a good strategy to attenuate liver fibrosis and that 5PEGIL-10 is the most effective conjugate.

Keywords: *Interleukin-10; PEGylation; Pharmacokinetic profile; Liver fibrosis; Macrophage*

Introduction

Liver fibrosis is a major cause of morbidity and death worldwide and represents a sustained wound-healing process in response to chronic injury, induced by chronic viral hepatitis, drugs or alcohol abuse, non-alcoholic steatohepatitis, autoimmune, parasitic, cholestatic or metabolic diseases [1,2]. The disease is characterized by an excessive deposition of collagens, mostly types I and III [1,3], which are mostly produced by activated hepatic stellate cells (HSC), the key pro-fibrogenic cells [1-3]. The end-stage is referred to as cirrhosis. A pharmacotherapy for this disease is unavailable [4,5]. As epidemiologic studies show that the prevalence of the disease will increase significantly in the coming years, a therapy for fibrosis is warranted [6].

During disease progression, the concerted actions of many cytokines control the activity of hepatic cells leading to a balance between pro- and anti-fibrotic activities. One of the few anti-fibrotic cytokines that is produced during fibrogenesis is interleukin-10 (IL-10).

IL-10 is expressed by different immune cell-types [7] and within the liver, it can be produced by nearly all resident hepatic cells [8]. It is a potent immunomodulatory cytokine that downregulates several proinflammatory cytokines in macrophages, including IL-1β and IL-6, and decreases the expression of MHC class II molecules [8,9]. *In vitro*, it downregulates collagen 1 and increases expression of matrix metalloproteases in HSC and thus promotes degradation of collagens I and III [10,11]. It is also postulated that IL-10 can prevent activation of quiescent HSC and cause apoptosis of activated HSCs [12]. In 2003, a clinical trial in patients with hepatitis B-induced fibrosis, revealed a downregulation of fibrotic parameters, but the study was abandoned due to a flare-up of viral activity [13].

The rapid renal clearance of IL-10 that results in a low plasma half-life is one of the limitations to use this cytokine for the treatment of liver fibrosis [14]. One way to avoid this effect is the chemical attachment of polyethylene glycol to the cytokine (i.e. PEGylation) [15]. PEGylation can enhance the therapeutical potency of peptides and proteins by prolonging their half-life in the bloodstream [16-18]. PEGylation may affect the biological activity in vitro but in vivo experiments have shown that the increased circulation time can compensate this [19]. IL-10 was recently PEGylated and showed better effects than unmodified IL-10 in controlling neuropathic pain [18]. However, although IL-10 is a relevant cytokine and PEGylation has already proven its value for many cytokines, studies examining the biological effects of PEGylated IL-10 in vivo are scarce. Only local administration has been examined [20], and the effects have never been explored in experimental models of disease. Also a study on the optimal size of the PEG molecules, which greatly determines the circulation time and receptor binding, is completely lacking.

In the present study, we therefore modified IL-10 with two different sizes of PEG (5kDa, 20kDa) and examined the circulation times and biological effects in a CCl_4-induced acute model of liver fibrosis in mice. Our results show improved effects of PEGylated IL-10 compared to IL-10 on the fibrotic process and macrophages within the fibrotic liver. In particular its modification with the 5Kda PEG molecule yields a long circulating and effective antifibrotic compound, which provides a strong basis for further studies on modified IL-10 as a therapeutic compound. Our studies also indicate that the effects of PEG-IL10 are mediated by a subset of macrophages within the liver.

Material and methods

IL-10 PEGylation and Characterization

IL-10 PEGylation. Five microgram of recombinant human IL-10 (rhIL-10, PeproTech EC Ltda., UK) was brought to a concentration of 50μg/mL in 1× PBS buffer (0.01M), pH 7.2–7.4. PEGylation, via the amine groups, was done overnight at 4°C by adding 6.4 μL of 12.5 mg/mL 5 kDa mPEG-NHS or 50 mg/mL 20 kDa mPEG-NHS (NANOCS, USA; ratio 1:50), as described before [21]. The reaction was followed by 24 h dialysis against 1× PBS, using suitable dialysis membranes (Harvard Apparatus, Holliston, MA) to remove any unreacted PEG. Presence of unreacted IL-10 was checked in Western blots after 10% SDS-polyacrylamide gel electrophoresis and subsequent silver staining according to Morrissey [22]. PEGylation was confirmed by barium–iodine PEGstaining of SDS-PAGE gel. Briefly, 200 ng of IL-10 and the PEGylated forms of IL-10 were loaded to the 10% SDS-PAGE. After the running, the gels were rinsed with water and fixated with perchloric acid (0.1M) for 15 min and then washed with water. Subsequently, the gel was incubated in barium chloride (5%) for 10 min and developed using tritisol iodine solution (Sigma, Cambridge, UK). The gels were photographed using GBox (Syngene, Cambridge, UK). Products were stored at −20°C. The protein concentrations used in the reactions were too low to be detected in protein assays and therefore the final protein concentrations were calculated based on the initial amount of IL-10 added to the reaction mixture.

Cells and cell line

RAW 264.7 – Mouse embryonic cell line. RAW 264.7 cells (ATCC, TIB-71) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Belgium) containing 10% fetal bovine serum (FBS, Biowhittaker, Belgium), 60 μg of gentamicin (Gibco, Invitrogen), 2 mM of L-glutamin (Gibco, Invitrogen) and 0.48 M of L-arginine (Sigma, USA). Cells were used for experiments until a maximum of 20 passages.

Assessment of bioactivity in vitro

RAW 264.7 cells ($1.5x10^5$ cells/well) were cultured overnight in 96 wells plates (Costar®, Corning Inc., USA). First, the cells were preconditioned for 1 h at 37°C in 0.2 mL of FBS free medium. After this, cells were preincubated for 30 minutes with 50 ng/mL of IL-10, 5PEGIL-10 or 20PEGIL-10 in 0.1mL FBS-free medium containing 0.5% normal mouse serum. At t = 0, 25 ng/mL of LPS (E.coli, List Biological Laboratories, INC., USA) was added to the wells. Control cells were not incubated with LPS. At t = 6 h, TNF-α levels in the culture media were determined using a sandwich TNF-α ELISA kit (BD PharMingen, USA). Assays were performed with two different batches of 5 and 20PEG-IL-10 and in triplicate for each batch.

Animals and experimental model of liver fibrosis

Pathogen-free male C57BL/6 mice weighing 20 to 22 g, purchased from Harlan (Zeist, The Netherlands) were used in this study. Animals were housed under laboratory conditions and received *ad libitum* normal diet. The experimental protocols for animal studies were approved by the Ethical Animal Committee of the University of Groningen. To induce acute liver fibrosis, mice received a single intraperitoneal injection of CCl_4 (1 mL/kg in olive oil). Animals were either used for distribution studies or effect studies.

Distribution studies

IL-10, 5PEGIL-10 and 20PEGIL-10 were labeled with ^{125}I according to standard methods [23]. Before each experiment, free iodine was removed with a PD-10 column (GE Healthcare). 72 h after the CCL_4 injection (1 mL/kg in olive oil), mice (n = 3 per group per time-point) were anesthetized with O2/N2O/isoflurane and received an i.v. injection (penile vein) of [^{125}I]IL-10, [^{125}I]5PEG-IL-10 and [^{125}I]20PEG-IL-10 (± 1,000,000 cpm per animal, in saline). After 15 min, 3 h and 24 h, blood and livers were harvested to check the amount of radioactivity. A gamma counter (Riastar Gamma Counting System; PerkinElmer Life and Analytical Sciences, Boston, MA) was used to measure the total amount of radioactivity in blood samples and livers. Liver samples were corrected for blood-derived radioactivity, as previously described [24].

Effect studies

To assess the effects of IL-10 and PEGylated forms of IL-10, on fibrotic parameters *in vivo*, we injected, via penile vein, CCL_4 treated-mice with vehicle (PBS), IL-10, 5PEGIL-10 or 20PEGIL-10, all in a dose of 10μg/kg/day (n = 6 per group), 24 h and

48 h after CCL4 administration. Subsequently, animals were sacrificed 24 h after the last injection (72 h after CCL_4 administration). Organs were taken out and prepared for further analysis.

Immunohistochemistry. To access the amount of activated HSCs, livers were fixated in 4% formalin and embedded in paraffin. After deparaffinization, 4 μm sections were stained for α-SMA according to standard methods using a monoclonal antibody (Sigma-Aldrich), and a biotinylated secondary anti-mouse IgG antibody. Staining was visualized using the Vectastain Elite ABC Mouse IgG kit (Vector Laboratories, Burlingame, CA). Expression of type III collagen, macrophages and antigen-presenting cells was examined in aceton fixated cryostat sections (4μm) according to standard indirect immunoperoxidase methods [25] with goat anti-collagen III (SouternBiotech, USA), rat anti-F4/80 (Serotec) or rat anti-MHC class II (Santa Cruz Biotechnology, USA) IgGs, respectively. Staining was quantified by image analyzing techniques, using Multiple Alignment Analysis (MIAs). The results were given as percentages of positive-stained areas divided by the total area of the section.

Real-Time PCR. Isolation of total RNA from mice livers was performed using the RNeasy kit (QIAGEN, Hilden, Germany), and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, NC). The reverse transcriptase reaction was done using random primers (Promega). The transcription levels of mice IL-1β (forward 5'GCCAAGACAGGTCGCTCAGGG3' and reverse 5'CCCCCACACGTTGACAGCTAGG3') and IL-6 (forward 5'TGATGCTGGTGACAACCACGGC3" and reverse 5'TAAGCCTCCGACTTGT3') were detected by quantitative real-time PCR methods with SensiMix™ SYBR kit (Bioline) on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA).

Quantification of data was performed via comparative ΔΔ Ct calculation (β-actin as housekeeping gene), and the gene expression levels in livers from CCL_4 treated animals were set at baseline.

Western blotting. Proteins were isolated from frozen tissue (30-40mg) and samples (100 μg) were separated in a 10% sodium dodecyl sulphate-polyacrylamide (SDS) gel. Subsequently, proteins were transferred to a PVDF membrane, according to standard procedures, for 2 h. β-actin (Sigma, A5316, 1:5000) was used as a control for equal loading of protein samples. Primary antibodies were diluted and added as follows: YM1 (R&D systems, AF4226, 1:200); IFR5 (Proteintech Europe, 10541-1-AP, 1: 800); and FOXP3 (eBiosciences, 13-5773-82, 1:100) and the membranes with the primary antibodies were incubated at 4°C overnight, then washed for 30min with 0,1% Tween in TBS and incubated with secondary antibodies.

Computerized densitometry was used to measure the intensity of the bands and the relative intensity was determined through the ratio of the intensity of primary ab:β-actin (GeneSnap, SYNGENE).

Statistical analysis. Results were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni test. P< 0.05 was considered statistically significant.

Results

Modification and characterization of PEGylated forms of hIL-10

Two forms of PEG (5 kDa and 20 kDa) were covalently linked to lysine groups of IL-10 as illustrated in Figure 1A. SDS-PAGE was performed to analyze the molecular weight of the modified proteins (Fig. 1.B). IL-10 showed a band at ± 37 kD, corresponding to its dimeric (active) form. A shift in MW was shown by 5PEG-IL-10, with a major band at 75-80KDa plus a minor band at ±120 kDa. The 75– 80 kDa band was also positive after PEG-staining (Fig. 1C), whereas the 120 kDa band was not. We conclude that the 120 kDa band is a multimeric form of IL-10. Only the homodimeric form with two essential linking disulfide bonds is biologically active [26]. 20PEGIL-10 could not be visualized on the silver staining gel probably because the PEG molecules were shielding the IL-10 molecules, but data showed the complete absence of unmodified IL-10 in the synthesized product. Furthermore, in the PEG-stained gel, one major band was visualized, at 100–120 kDa, indicating a major shift in MW of IL-10 (Fig. 1C). No free IL-10 dimer was detected in the lanes containing 20PEGIL-10 (Fig. 1B), demonstrating that the reaction was complete. Collectively, the blots confirm the PEGylation of IL-10 with, respectively, 5 and 20 kDa molecules and also show that results obtained with 5PEGIL-10 and 20PEGIL-10 cannot be attributed to the presence of native IL-10 in the synthesized products.

TNF-α release by macrophage. To test whether the biological activity of IL-10 was affected by the chemical modification, *in vitro* anti-inflammatory effects of IL-10 and its PEGylated forms were assessed in LPS-stimulated RAW 264.7 cells. RAW cells showed a significant TNF-α release in response to LPS as compared to the control, as shown in fig. 2. This response could be inhibited by 29±6.5% by IL-10 ($p<0.001$), 41±5.6% by 5PEG-IL-10($p<0.001$) and 42±3.1% by 20-PEG-IL-10 ($p<0.001$) at a concentration of 50μg/mL for each compound. This indicates that the anti-inflammatory activity of IL-10 is preserved after the chemical attachment of PEG moieties.

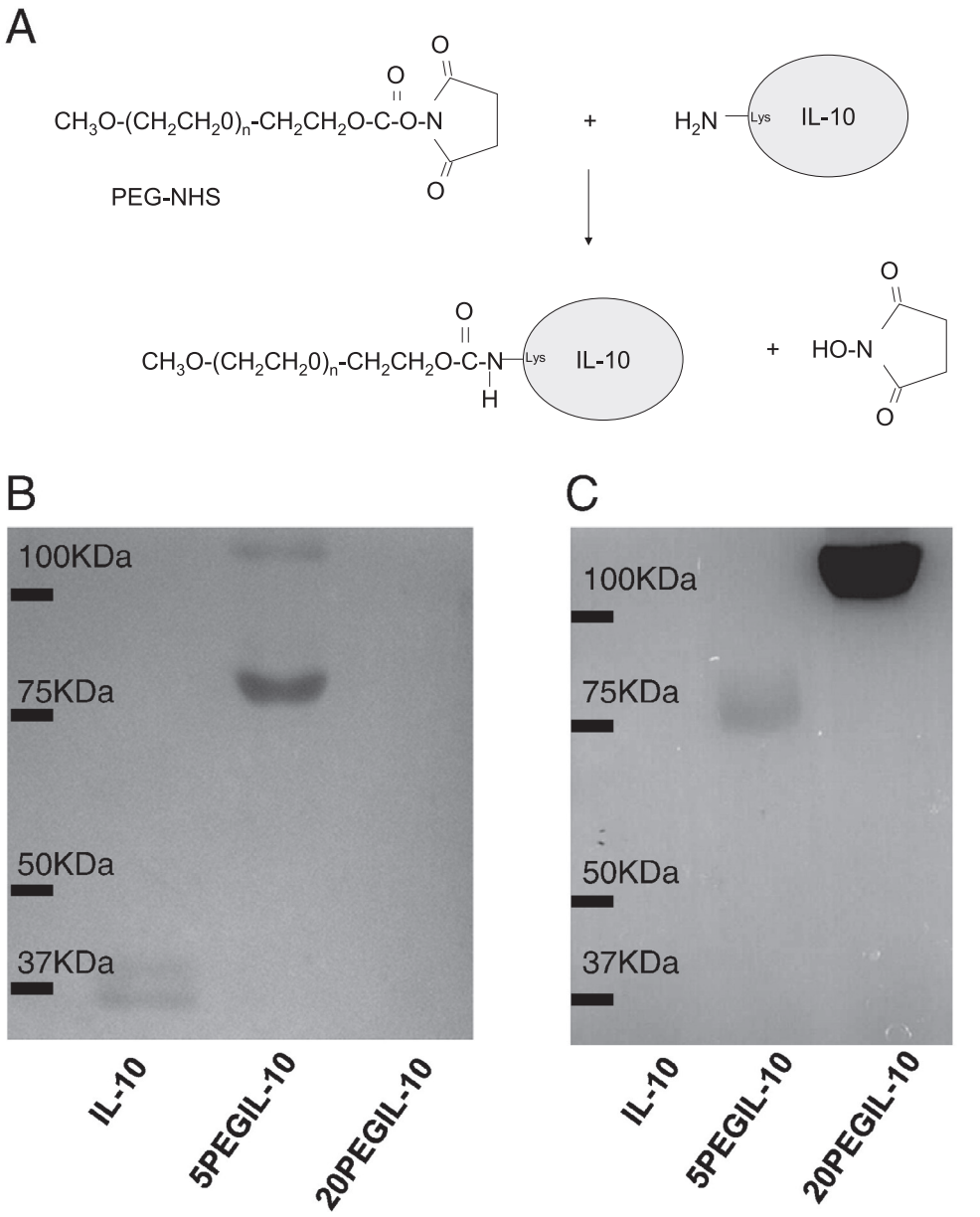

Fig. 1. *Schematic diagram of the synthesis of PEGylated IL-10. (**B**) Silver staining of SDS-PAGE of IL-10 and its PEGylated forms. Dimeric IL-10 (37KDa; **lane I**) was modified with 5 KDa PEG (**lane II**) and the new compound had an increased molecular weight (75-80KDa). The modification with 20 KDaPEG (**lane III**) was not detectable after silver staining, but the blot shows the complete absence of unmodified IL-10. (C) PEG-staining of SDS-PAGE of IL-10 and its PEGylated forms. PEG-positive bands were found at 75–80 kDa for 5PEGIL-10 and at 100–120 kDa for 20PEGIL-10.*

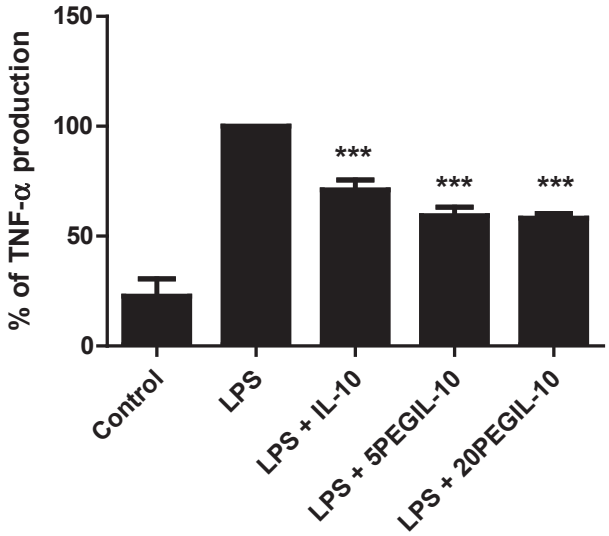

Fig. 2. *Effect of IL-10, 5PEGIL-10 and 20PEGIL-10 on TNFa release by RAW 264.7 cells in vitro. Cells were incubated with 50 ng of IL-10, 5PEGIL-10 and 20PEGIL-10, 30 min before a challenge with LPS (25 ng/mL). It can be seen that TNF-α production was significantly inhibited by all IL-10 compounds (*p< 0.001 compared to LPS-stimulated cells). Results are expressed as mean±SD (n = 3).*

***In vivo* studies**

Blood and liver accumulation of hIL-10 and its PEGylated forms. We compared the accumulation of [^{125}I]IL-10, [^{125}I]5PEG-IL-10 and [^{125}I]20PEG-IL-10 in livers and blood of CCl_4-treated mice in order to check if PEGylation was associated with an increased circulation time and liver accumulation of IL-10. As can be seen in figure 3A, only 6±1.0% of the initial dose of radiolabeled-IL-10 was found in the blood 15 min after i.v. injection. The amounts of both 5PEG-IL-10 and 20PEG-IL-10 (40± 7.5% and 44 ± 4.9%, respectively) in blood were significantly higher compared to IL-10 (P< 0.001) and concentrations of 5PEG-IL-10 and 20PEG-IL10 remained significantly (p<0.001) higher up to 24 h after administration compared to native IL-10, which was completely gone from the circulation at that time point. The difference between 5PEGIL-10 and 20PEGIL-10 was not significant. As shown in Figure 3.B, liver uptake of IL-10 and its PEGylated forms was not significantly different from each other at t = 15 min, but after 3 hours, both 5PEGIL-10 (8%, ± 1.9) and 20PEGIL-10 (9%, ± 1.2) displayed a higher liver accumulation compared to IL-10 (3%, ± 1.4) (P<0.01). 24 h after the i.v. injections, IL-10 was undetectable in the liver, while 5PEG-IL-10 and 20PEGIL-10 were still detected in liver tissue (P<0.01) (fig. 3.B). So, after PEGylation of IL-10 with either 5 or 20 PEG the circulation time is significantly improved and the levels within the fibrotic livers are significantly higher for a prolonged period of time.

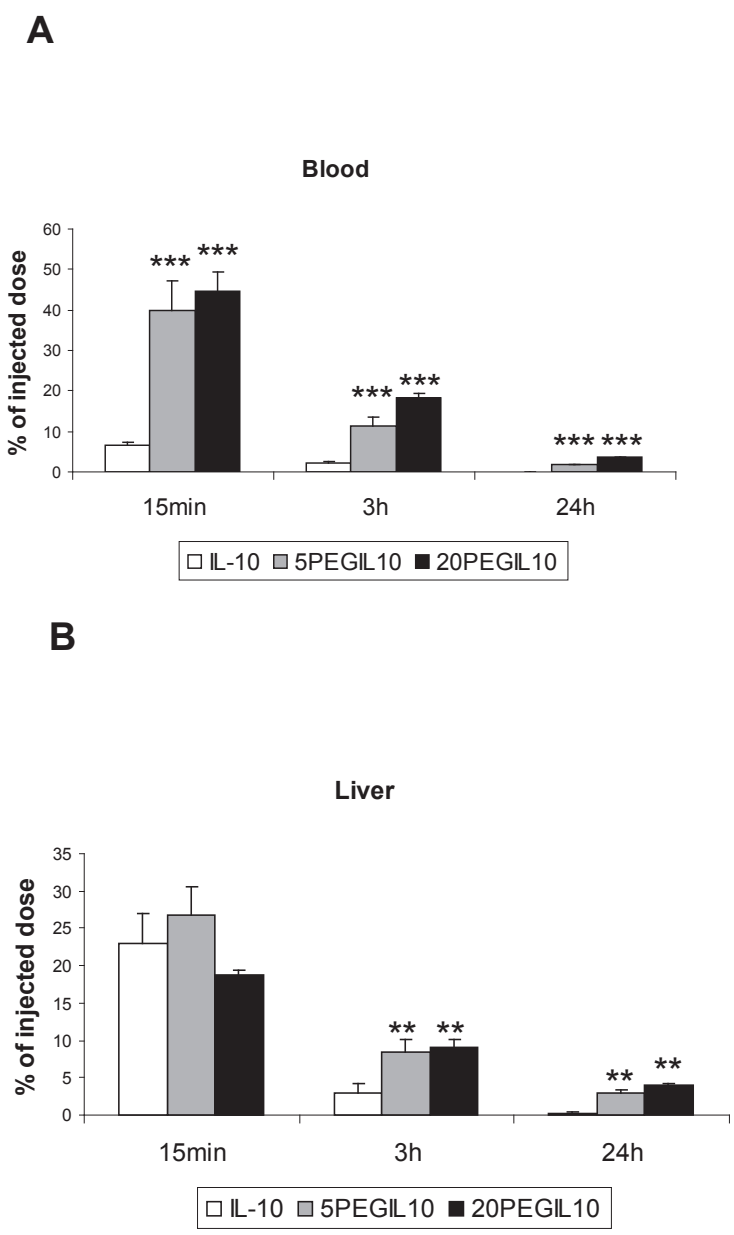

Fig. 3. *Biodistribution of [125 I]IL-10, [125 I]5PEG-IL-10 and [125 I]20PEG-IL-10, at various time-points after intravenous injection of tracer amounts in mice with CCL4-induced liver disease. (A) Results show an increased circulation time of [125 I]5PEG-IL-10 and [125 I]20PEG-IL-10 in the blood (**p<0.001); (B) Liver accumulation of all three compounds was similar at t = 15 min, but uptake at 3 h and 24 h of [125 I]5PEG-IL-10 and [125 I]20PEG-IL-10 was increased in the liver (*p<0.01).Results are expressed as mean±SD with n = 3/group/timepoint.*

Effects of PEGylated IL-10 in CCl4 treated mice.

Since the biological activities of IL-10 were not altered *in vitro* after PEGylation and the *in vivo* distribution of IL-10 to the liver was improved after attachment of PEG, we proceeded to study the *in vivo* effects of the two PEGylated forms of IL-10 relative to unmodified IL-10. For this purpose, we used an acute CCl_4 mice model, characterized by activation of HSC (assessed by α-SMA expression) and deposition of fibrilar collagen (assessed by collagen type III expression) in areas of CCL_4 induced cell damage, as depicted in figure 4A and B. We analyzed the effects of different compounds on liver histology using immunohistochemical markers for Kupffer cells (F4/80), antigen-presenting cells (APCs; MHC class II molecules expression), activation of HSC (α- SMA), and deposition of COLL III. The results are shown in figures 4 and 5. The group that received 5PEGIL-10 showed a lower collagen III deposition, although the difference was not significant ($p<0.06$) (Fig. 4A and 4C), which may be related to the short duration of the treatment (2 injections). Expression of α- SMA was nearly 50% lower ($p<0.05$) after administration of 5PEGIL-10 as compared to the PBS control, whereas administration of IL-10 or 20PEGIL-10 did not induce a significant effect compared to PBS-treated fibrotic mice (Fig. 4B and 4D).

IL-10 is reported to downregulate MHC class II molecules in myeloid cell types [27] which contribute to the anti-inflammatory activities of IL-10. MHC II expression in CCl_4-treated livers was found in damaged areas (see figure 5A). In these areas, a 64% decline in MHC class II expression was found in the 5PEGIL-10 treatment group ($p<0.01$) compared to the control group (fig 5A and 5C), whereas IL-10 or 20PEGIL-10 did not induce a significant effect. Within livers, MHC class II can be expressed by KC, T cells and HSC [28]. The number of CD4 positive cells in livers was very low and did not correlate with MHC II expression (data not shown). Alternatively, KC may be positive for MHC II after activation. Indeed, we found F4/80 staining in the same region, although much more cells were positive F4/80 than for MHC II. F4/80 expression did not change after treatment with IL-10 or its PEGylated forms (Fig. 5B and 5D), indicating no reduction in the number of KC cells, but a reduction in their activity.

IL-10 is also known to downregulate the expression of several pro-inflammatory cytokines [29]. Analysis of IL-1β expression levels with PCT methods (Fig 6) revealed a 47% and 51 % reduction in 5PEGIL-10 and 20PEG IL-10 treated groups respectively ($p<0.05$), whereas unmodified IL-10 did not significantly reduce IL-1β transcription, compared to the control group (Fig 6A). Transcription of IL-6 was significantly downregulated in all treated groups compared to the untreated control group ($p<0.05$).

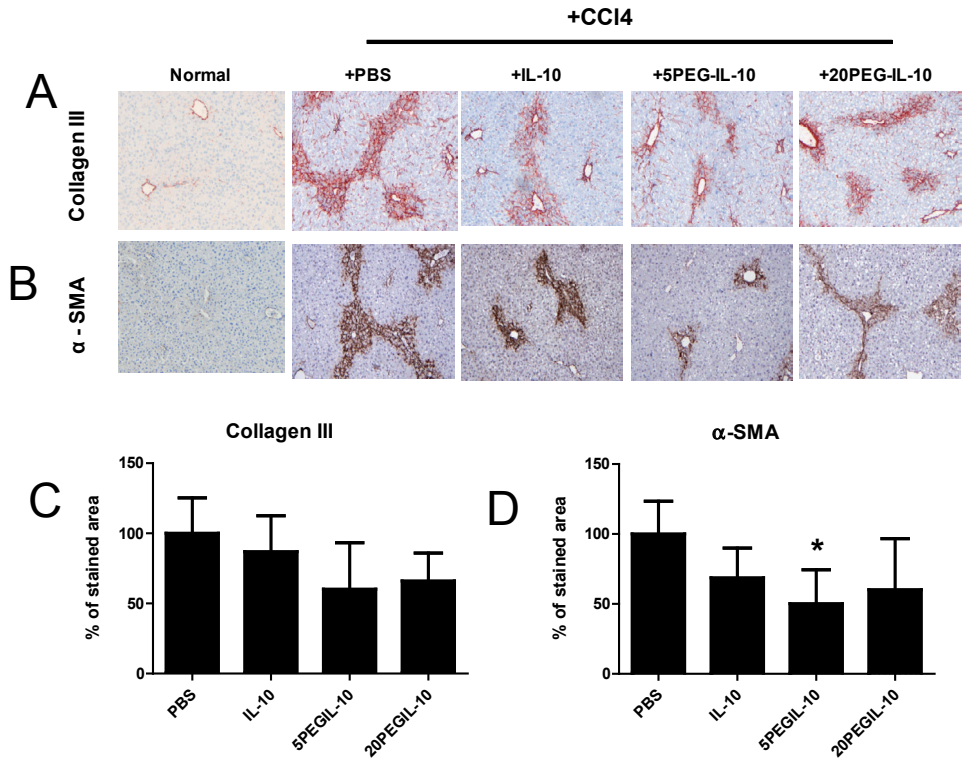

Fig. 4. *Antifibrotic effects of IL-10 and PEGylated IL-10 in CCl4-induced liver fibrogenesis in mice (A,B). Representative pictures of immunohistochemical staining for Collagen type III (A) and α- SMA (B) on liver cryostat sections. (C,D) Quantification of the positive stained area for Collagen III (C) and α- SMA (D) using the Olympus Cell D analysis program. Results are shown as mean ±SD (n = 6 per group). Analysis by one way ANOVA with Bonferroni post-hoc test revealed that the group treated with 5PEGIL-10 displayed a reduction in collagen III ($p<0.06$) and α- SMA (*$p<0.05$) staining as compared to the PBS group.*

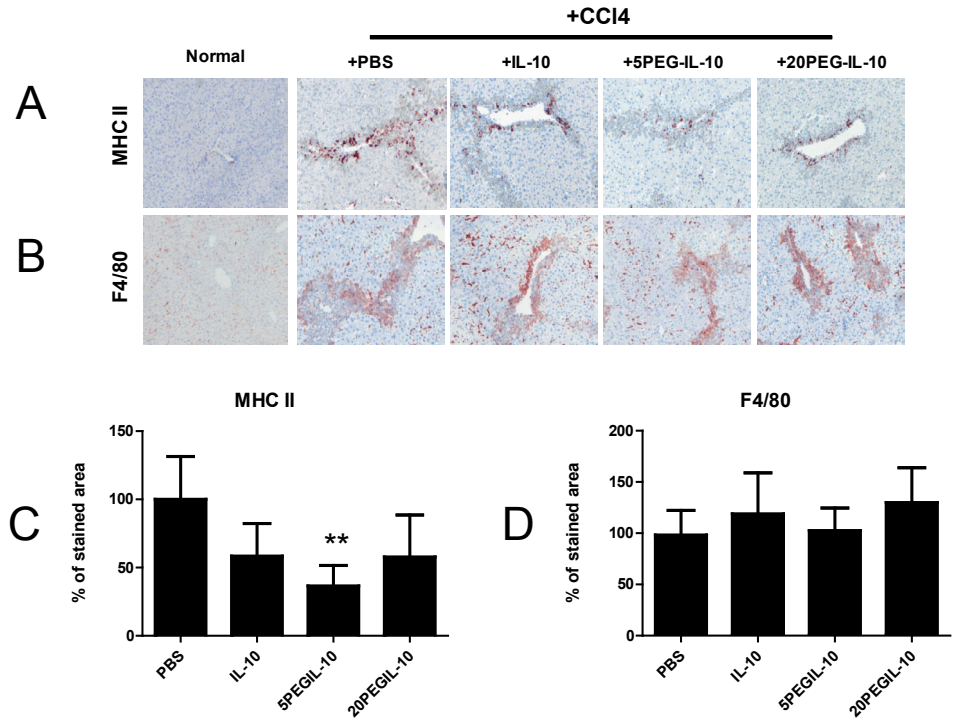

Fig. 5. *Effects of IL10 and PEGylated IL10 on MHC II expression and number of Kupffer cells. (A, B) Representative pictures of immunohistochemical stainings for MHC class II (A) and F4/80 (B) on mice liver sections. Animals received a single dose of CCL4 and were treated after 24 h and 48 h with PBS, IL-10, 5PEGIL-10 or 20PEGIL-10. (C, D) Quantification of the positive stained area for MHC II (C) and F4/80 (D) using the Cell D image analyzing software. Results are shown as mean ±SD (n = 5-6 animals per group). Analysis by one way ANOVA with Bonferroni post-hoc test revealed that only the group treated with 5PEGIL-10 had a significant reduction in MHC class II expression(*p<0.01). The area stained for F4/80 (D) did not significantly differ among the groups.*

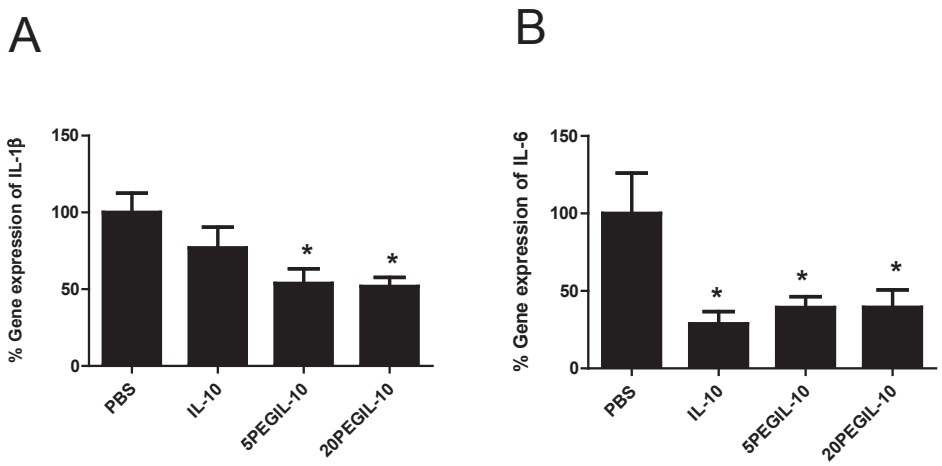

Fig. 6. *Effects of IL-10, 5PEGIL-10 or 20PEGIL-10 on the hepatic mRNA levels for IL-1β and IL-6. (A) IL-1β was significantly decreased in 5PEGIL-10 and 20PEGIL-10-treated groups as compared to the PBS-treated diseased mice. Unmodified IL-10 did not induce a significant decrease in IL-1β transcription. (*p<0.05) (B) IL-6 gene expression was significantly downregulated by IL-10 and both PEGylated forms of IL-10 (*p<0.05). Results are shown as mean± SD (n = 5-6 animals per group) and statistical analysis was performed by one way ANOVA with Bonferroni post-hoc test.*

Effects of IL-10 and PEGylated forms of IL-10 on macrophages of CCl4 fibrotic livers. Recent studies indicate that macrophages are cells that can be polarized into a M1 (classic) or a M2 (alternative) phenotype according to the microenvironment and IL-10 was found to modulate this phenotypic switch [30]. M1 macrophages, characterized by high production of proinflammatory cytokines, mediate the resistance against pathogens and contribute to tissue destruction. These cells can be identified by Interferon-regulatory factor 5 (IRF5) [31]. Alternatively activated macrophages (M2) can be identified by the marker YM1 and produce high amounts of anti-inflammatory cytokines, and they promote tissue repair and remodeling and tumor progression [30]. Recently, the expression of forkhead lineage-specific transcription factor FOXP3, which is a well known marker for regulatory T cells (Treg cells), was also shown to identify a sublineage of macrophages found in several organs, including the liver. $FOXP3^+$ confers suppressive functions to macrophages [32]. In the present study, there were no significant differences in IFR5 and YM1 markers between the different groups of mice (Fig. 7A, B, C), but the groups treated with 5PEGIL-10 or 20PEGIL-10 showed a significant increase ($p<0.01$) in FOXP3 expression (Fig 7A, D). These cells might therefore mediate the anti-fibrotic effects seen for PEGylated IL-10. These data show that the PEGylated forms of IL-10 modulate the phenotype of macrophages within the liver.

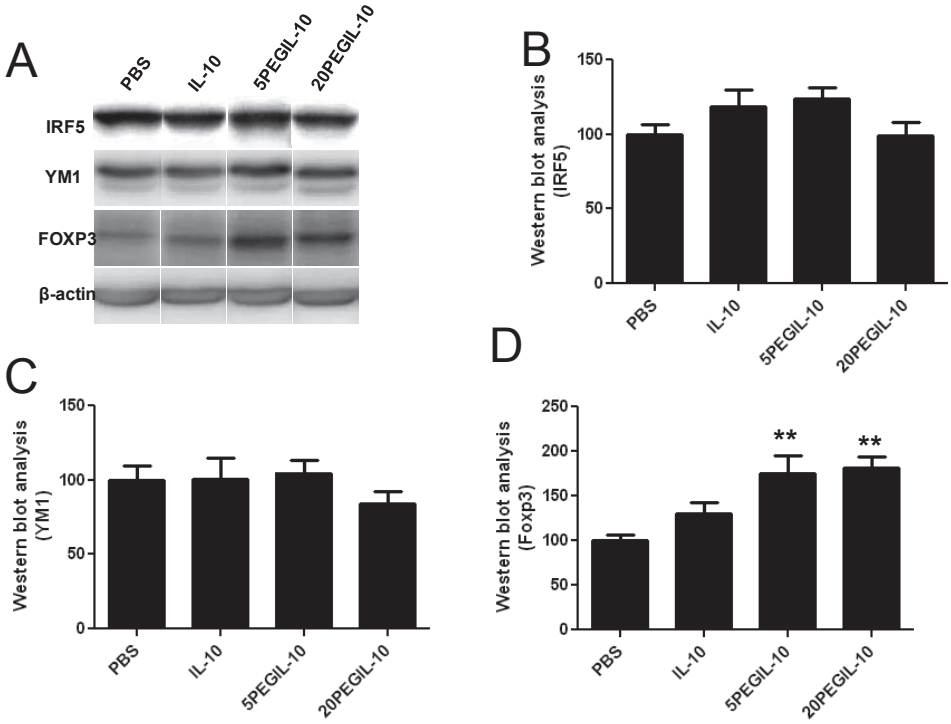

Fig. 7. *Western blot analysis of macrophage profiles in fibrotic livers treated with IL-10 or PEGylated forms of IL-10 (A). IRF5 (M1 marker) did not significantly change in any of the groups (B) nor did YM1 expression (M2 marker) (C). Foxp3 expression (deactivated macrophages-M2c) was significantly upregulated in both 5PEGIL-10 and 20PEGIL-10 -treated groups ($p<0.01$) (D).*

Discussion

Interleukin-10 is a potent anti-inflammatory cytokine that is able to decrease the expression of many pro-inflammatory cytokines in several cell types and shift the maturation of immune cells [7]. In addition to that, IL-10 also induced significant anti-fibrotic effects in several studies [13,33,34], but a clinical trial with IL-10 in cirrhotic patients was unsuccessful [13]. Similar to many others interleukins, IL -10 has a short half-life in plasma due to rapid renal clearance, which is an important factor that limits its therapeutic use [14,18]. Polyethylene glycol (PEG)- conjugation is a widely used strategy to improve the pharmacokinetic profile of proteins. PEGylation prolongs the half-life of proteins by preventing their renal clearance and preventing enzymatic proteolysis in serum [15,35]. It also reduces the immunogenicity of a conjugated protein. On the other hand, the improvement in the pharmacokinetic profile of a protein often comes at cost of a reduced binding capacity to its receptor [35].

To date, there is very little information on the effects of PEGylation on the pharmacokinetic profile and therapeutic effects of IL-10. Therefore we now examined the biodistribution in fibrotic mice. Studies about the pharmacokinetics of IL-10 have been conducted in healthy individuals [36] and in renal disorder animal models [37] and in the bile duct ligated (BDL) rat model for liver fibrosis [14]. Although PEGylated IL-10 has been previously produced and tested against neuropathic pain, it was used locally and the systemic distribution has not been accessed [18].

Therefore, we PEGylated IL-10 and tested its antifibrotic effects in vivo. Our data show a longer circulation time and higher accumulation in fibrotic livers. In the present study, we PEGylated IL-10 with two different sizes of PEG: 5KDa and 20KDa to study which one was the most appropriate to optimize the ratio biodistribution/effects. PEGylation was performed by coupling of PEG to the secondary NH2 groups of lysine. There are 13 lysine residues present in the IL- 10 molecule but not all of them are accessible for chemical modification. It was not possible to quantify the number of PEG molecules coupled to IL-10 using mass spectrometry because of rapid degradation during analysis.

Coupling of PEG to IL-10 via lysine groups may lead to a decreased biological activity due to blocking of its binding properties to the IL-10 receptor [38]. Therefore, we used an in vitro system to assess anti-inflammatory IL-10 properties after the PEGylation. The response of RAW cells on LPS can be downregulated by IL-10 [39]. A significant inhibition of LPS-induced TNF-α production was induced by IL-10 and also by both 5PEGIL-10 and 20PEGIL-10, all at equal concentrations, indicating that the bioactivity of IL-10 was preserved after PEGylation.

Subsequently, we examined the circulation time of (PEG) IL-10 in the blood of animals with CCl_4-induced liver damage. PEGylated forms of IL-10 were cleared much slower than IL-10 itself; they circulated longer and accumulated significantly more over time within the liver. 24 h after the initial dose, there was neither IL-10 in the circulation nor in the liver anymore, but we still could trace back 5PEGIL-10 and 20PEGIL-10 in the blood and livers. These data confirm that a longer circulation time corresponds with a higher liver uptake and a longer residence time in this target organ, which is important considering the fact that liver fibrosis is a chronic disease.

As PEGylated forms of IL-10 showed biological activity *in vitro* and a prolonged circulation time and higher liver accumulation in vivo, we continued by assessing its antifibrotic effects in vivo. A single dose of CCL_4 is known to lead to extensive necrosis and a consequent inflammatory response, which lead to HSC activation and local collagen deposition in the liver after 72 h. The effects of compounds on the fibrogenic process can be readily accessed in this situation. The main findings after immunohistochemical analysis showed a significant decrease in the number of activated hepatic stellate cells (α-SMA staining) and a decrease in APCs cells (MHC class II staining)

by 5PEGIL-10. IL-10 and 20PEGIL-10 did not induce significant changes in these parameters although IL-10 was previously reported to prevent activation and cause apoptosis of HSC [12]. Although 5PEGIL-10 induced a 50% reduction in αSMA positive cells (reflecting activated HSC), this caused only a slight reduction in collagen III deposition. Apparently the treatment was too short to induce a significant effect on scar tissue formation.

During fibrogenesis, antigen presenting cells (APCs) in the liver display an upregulation of MHC class II molecules [40]. Activated APCs promote intrahepatic inflammation, which contribute to the perpetuation of the fibrogenic process. IL-10 was reported to be able to downregulate MHC class II expression during liver fibrosis [40,41], but in the present study, only 5PEGIL-10 significantly decreased MHC II expression in liver cells. Next to this downregulation of MHC II expression, real-time PCR showed a downregulation in the transcription of two pro-inflammatory cytokines: IL-6 was significantly decreased in all treatment groups, while the PEGylated forms of IL-10 also attenuated IL-1β production. The effect of (PEG)-IL-10 seems therefore, at least partly, to be mediated by an effect on antigen presenting cells, which are mostly F4/80 positive macrophages. It is known that macrophages, next to HSC, play an important role during fibrogenesis and several subpopulations with sometimes opposing activities have been identified in recent years [42,43]. Elucidation of the mechanisms behind this macrophage plasticity is now seen as a main topic to understand the process of fibrogenesis [43]. IL-10 is known to down regulate macrophage activities [44] (see also Fig 2), and can also affect the phenotype of macrophages by inducing an M2c–phenotype [30]. These regulatory M2c macrophages have potent anti-inflammatory effects [32], but their role in fibrosis is unknown. Recently, the marker for regulatory T cells- i.e. forkhead box P3 (Foxp3) [45] was found to be also expressed by suppressive/regulatory macrophages in several organs, including the liver, but the role of Foxp3+ macrophages during fibrogenesis in vivo was never described. The number of Foxp3 positive cells was strongly increased in human patients with hepatitis C virus infections [46] and several other hepatic inflammatory diseases [47] and it was found that these cells produced TGFβ and IL-10 thereby suppressing the immune response [48]. The studies of Herbert et al.[46] indicate that TGFβ and IL-10 are both required to achieve an immunosuppressive effect during schistosomiasis, so the anti-inflammatory and anti-fibrotic effects of IL-10 seen in our studies might even be further enhanced upon co-administration with TGFβ. This remains to be elucidated. In the present study, we showed that 5PEG–IL10 induced a selective upregulation of Foxp3 expression, which was paralleled by a down regulation of proinflammatory and profibrotic activities within the the livers of 5PEG– IL10-treated mice. Our data show that PEG–IL-10 exerts an effect on these regulatory macrophages and suggest that these cells have antifibrotic effects.

In conclusion, we prepared different PEGylated forms of IL-10 which maintained their biological activities and showed a better pharmacokinetic profile in the acute mice model of liver fibrosis compared to unmodified IL-10. Modification with low molecular weight PEG molecules yields the best results: 5PEGIL-10 showed the best antifibrotic effects in vivo when compared to unmodified IL-10 or 20PEGIL-10. The antifibrotic effects of this study can be induced either by a direct effect on HSCs or indirectly via effects on macrophages, also demonstrated in this study.

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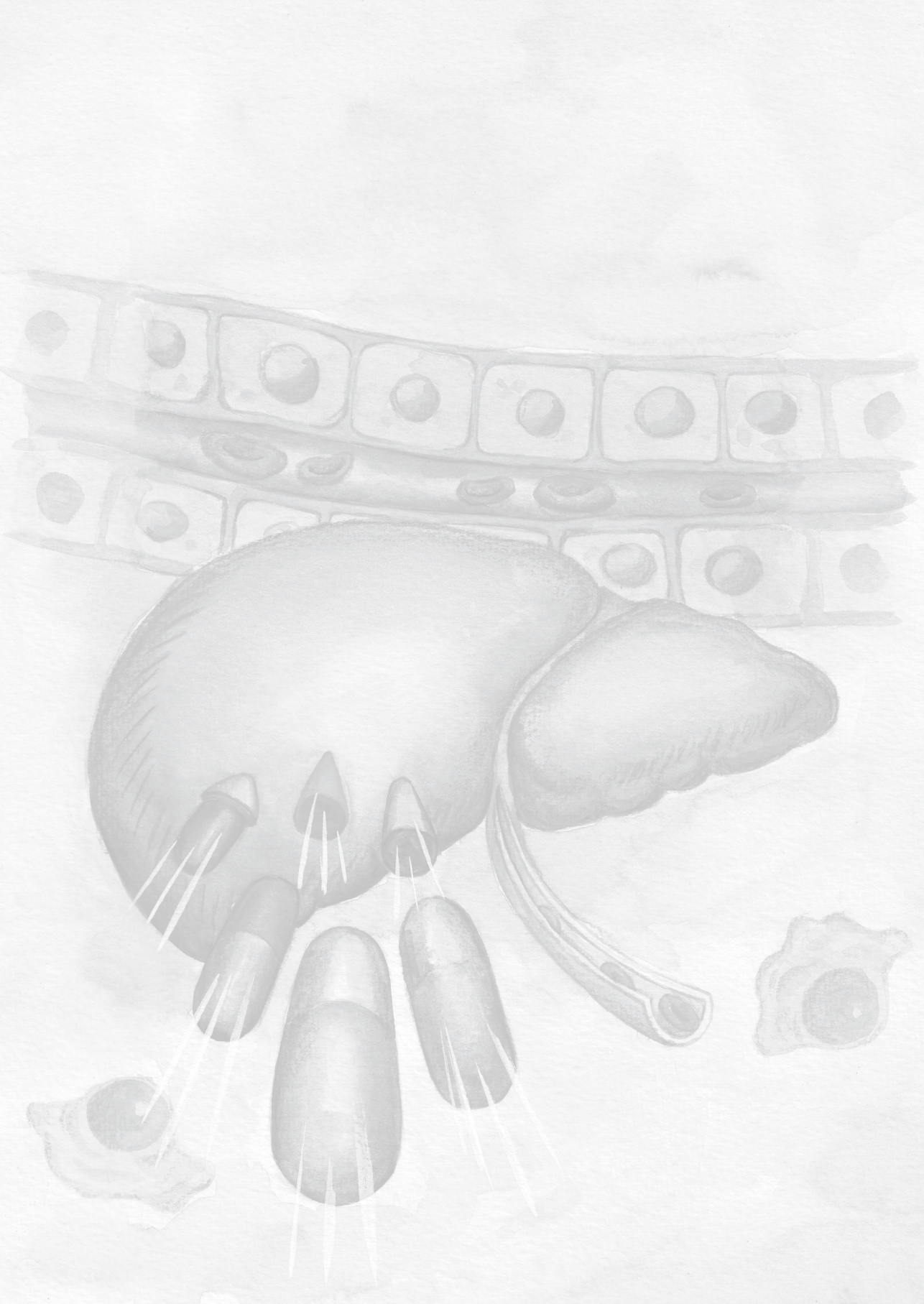

Chapter 4

IL-10 exerts a dual effect on fibrogenesis by affecting the macrophage phenotype and hepatic stellate cells

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Abstract

Interleukin-10 (IL-10) is known to have potent anti-inflammatory actions and some studies pointed out its antifibrotic properties, while others found that IL-10 has profibrotic activities. As IL-10 receptors are found on different cell types, we hypothesized that different cells can respond differently to IL-10. Our aim was therefore, to modify IL-10 with a cyclopeptide (PPB) that binds specifically to the PDGFβ-receptors, known to be upregulated in activated Hepatic Stellate Cells (HSC). Thus we delivered IL-10 to this cell type within the fibrotic liver. The biological activity of IL10 was preserved after conjugation as assessed by inhibition of TNF-α production by macrophages. *In vivo* studies in CCl_4 – induced chronic fibrogenesis in mice revealed a significant increased deposition of collagen type I in the group treated with IL10-PEG-PPB as compared to untreated or IL-10-treated groups. TIMP-1 protein and gene expression analysis revealed an upregulation only in the IL10-PEG-PPB-treated group. In contrast, MMP-13 expression was increased only in the IL-10-treated group as compared to control. The marker for M2 macrophages (YM1) was upregulated in the group treated with IL10-PEG-PPB, while the marker for M1 macrophages (IRF5) was downregulated. *In vitro* studies, using RAW 264.7 cells, confirmed that IL-10 only stimulates MMP-13 production in M1-polarized macrophages. Our data therefore suggest that IL-10 affects M1 macrophages with an antifibrotic phenotype while specific targeting to HSC induces a profibrotic response, possibly via induction of M2 macrophages. This illuminates the complex role of IL-10 during fibrogenesis.

Keywords: *Interleukin-10, drug targeting, hepatic stellate cells, liver fibrosis, macrophages.*

Introduction

Liver fibrosis is a wound-healing response after chronic hepatic injury, characterized by excessive deposition of extracellular matrix (ECM) and is an important cause of morbidity and mortality worldwide [1][2][3]. Chronic liver injury leads to activation of hepatic stellate cells (HSC) that are key players in the process of fibrogenesis by producing an excess of extracellular matrix (ECM) components [4]. Various cytokines are involved in the regulation of the balance between fibrogenesis and fibrolysis in the liver. In particular interleukin-10 (IL-10) has been considered as a cytokine that affects this balance [5].

IL-10 is produced by a number of cell types, including T and B cells, keratinocytes and macrophages [6]. In the liver, Kupffer cells, hepatocytes and hepatic stellate cells (HSC) are known sources of IL-10 [7]. IL-10 has been described as a potent immunosuppressor and anti-inflammatory cytokine via downregulation of monocyte/macrophage activity [8]. IL-10 is also endowed with direct antifibrotic effects in HSC as shown in *in vivo* models of liver fibrosis [9][10]. In 2003, Nelson *at al.* described that IL-10 treatment of patients with hepatitis B-induced liver fibrosis led to a decrease in fibrosis score, but the clinical trial was abandoned due to a subsequent increase in the virus burden in these patients [11]. This latter effect was probably due to the immunosuppressive actions of IL-10.

One of the explanations for the role of IL-10 in the resolution of liver fibrosis is related to up-regulation of matrix metalloproteinase-13 (MMP-13) and down-regulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) [12,13]. MMP-13 is responsible for the degradation of collagen I and III [14-17] and is mainly secreted by scar-associated macrophages during the resolution phase of liver fibrosis [18]. TIMP-1 is secreted mainly by activated HSC and is responsible for the inactivation of MMP-13 and the subsequent increased deposition of extracellular matrix (ECM). It is also involved in the proliferation and prevention of apoptosis of HSC [19,20]. Another possible explanation for the antifibrotic properties of IL-10 is that its anti-inflammatory effects could result in less fibrogenesis [14], but these results seem to be controversial because IL-10 is considered to be a Th2 cytokine which generally leads to profibrotic activities [21,22]. Further adding to the controversial properties of IL-10; pro-fibrotic effects were found in several organs like skin, kidney and lungs after IL-10 treatment, which was associated with polarization of macrophages to an alternative phenotype (M2) [23][24]. M2 macrophages are endowed with profibrotic activities via the production of TGFβ [25][26]. These cells also produce significant amounts of IL-10, contributing to the increased intrahepatic production of this cytokine during fibrosis [27].

Some studies showed that IL-10 causes apoptosis and inactivation of HSC *in vitro* and *in vivo* [28-30] and in 2007 Rachmawati et al targeted IL-10 to the M6P/IGFII receptor on HSC and obtained an antifibrotic effect in rats, but the study concluded that the targeting was also reaching Kupffer cells and endothelial cells, via

scavenger receptors [31]. In 2011, Bansal and co-workers specifically targeted INFγ to the PDGFβ-receptor expressed on activated HSC using a cycle peptide (PPB) [32].

We now aim to unravel the direct role of IL-10 in HSC during fibrogenesis. For this reason we chemically modified IL-10 with the PDGFβ-Recptor binding peptide PPB to target IL-10 to activated HSC. The conjugate (IL10-PEG-PPB) was characterized *in vitro* and its biological effects after conjugation were explored *in vivo* during CCl4-induced fibrogenesis in mice. The present study shows profibrotic effects of IL-10 after specific delivery to HSC *in vivo* which was associated with a significant increased influx of the M2 subset of macrophages in these livers. Our study illustrates the role of IL-10 in the cellular communication between HSC and macrophages during liver fibrogenesis *in vivo*.

Material and methods

iL10-PEG-PPB synthesis and western blot characterization. Five microgram of recombinant human IL-10 (rhIL-10, PeproTech EC Ltda., UK) was brought to a concentration of 50 μg/mL in PBS buffer, pH 7.2, and was reacted with 13 nmol of maleimide-PEG-succinimidyl carboxy methyl ester (MAL-PEG-SCM, 2kDa, Creative PEGworks, Winston Salem, NC) for 2 h followed by overnight dialysis at 4°C. The product was then incubated overnight at 4°C with PPB-ATA (26 nmol) in a solution containing hydroxylamine. After the reaction, PPB-PEG-IL-10 was dialysed against PBS for 24 h and stored at -20°C. Conjugation was confirmed using a 10% sodium dodecyl sulphate-polyacrylamide (SDS) gel where 0.1 μg of IL-10 and IL10-PEG-PPB were loaded. Proteins were transferred to a PVDF membrane, according to standard procedures, for 1 h. Primary antibodies were diluted and added as follows: rabbit polyclonal anti-IL-10 IgG (Santa Cruz Biotechnology, 1:200) and rabbit polyclonal anti-PPB IgG (1:1000; custom-made, Harlan) and the membranes with the primary antibodies were incubated at 4°C overnight, then washed for 30 min with 0,1% Tween in TBS and incubated with secondary antibodies.

Computerized densitometry was used to measure the intensity of the bands (GeneSnap, SYNGENE).

RAW 264.7 – Mouse embryonic cell line. RAW 264.7 cells (ATCC, TIB-71) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Belgium) containing 10% fetal bovine serum (FBS, Biowhittaker, Belgium), 60 μg/mL gentamicin (Gibco, Invitrogen), 2mM of L-glutamin (Gibco, Invitrogen) and 0.48 M of L-arginine (Sigma, USA). Cells were used for experiments until a maximum of 20 passages.

Assessment of bioactivity of constructs *in vitro*

RAW 264.7 cells ($1.5x10^5$ cells/well) were cultured overnight in 96-wells plates (Costar®, Corning Inc., USA). First, the cells were preconditioned for 1 h at 37°C in

0.2 mL of FBS-free medium. After this, cells were preincubated for 30 minutes with 50 ng/mL of IL-10 or PPB-PEG-IL-10 in 0.1 mL FBS-free medium containing 0.5% normal mouse serum. At t = 0, 25 ng/mL LPS (E.coli, List Biological Laboratories, INC., USA) was added to the wells. Control cells were not incubated with LPS. At t = 6 h, TNF-α levels were determined in culture media using a sandwich TNF-α ELISA kit (BD PharMingen, USA). Experiments were performed with two different batches of IL-10 and PPB-PEG-IL-10 and all experiments were done in triplicate.

Animals and experimental model of liver fibrosis

Pathogen-free male BALB/c mice weighing 20 to 22 g, purchased from Harlan (Zeist, The Netherlands) were used in this study. Animals were housed under laboratory conditions and received *ad libitum* normal diet. The experimental protocols for animal studies were approved by the Ethical Animal Committee of the University of Groningen. To induce chronic liver fibrosis, mice received intraperitoneal injections of CCl_4 (0.5 mL/Kg in the first week; 0.8 mL/Kg in the second week and 1 mL/kg in the remaining weeks, all in olive oil). For the effect studies, animals received seven injections (daily in the last week) of PBS, IL-10 or IL10-PEG-PPB (10 μg/Kg/day) and mice were sacrificed 24 h after the last CCl_4 injection.

Effect studies

Immunohistochemistry. To assess the effects of IL-10 and PPB-PEG-IL-10 on fibrotic parameters *in vivo*, we injected, via penile vein, CCl_4- treated BALB/c mice with vehicle (PBS), IL-10 (10 μg/kg/day/) or PPB-PEG-IL-10 (10 μg/kg/day) (n = 6 per group). Seven injections in total, daily in the last week of CCL_4 administration (week 8), were given to each animal. Subsequently, animals were anesthetized with O2/N2O/isoflurane and sacrificed 24 h after the last injection (8 weeks after the first CCL_4 administration). Organs were taken out and prepared for further analysis. To access the expression of type I collagen, 4 μm cryostat liver sections were fixated in acetone and stained using indirect immunoperoxidase methods. Briefly, sections were incubated for 1 h with goat anti-collagen I (Southern Biotech, 1310-01, 1:100) IgG. After that, sections were incubated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. Appropriate secondary and third antibodies were applied and the antibodies were visualized with 3-amino-9-ethylcarbazole (AEC, Sigma). Staining was quantified by image analyzing techniques, using Multiple Alignment Analysis (MIAs). The results were given as percentages of positive-stained areas divided by the total area of the section.

Real-Time PCR. Isolation of total RNA from mice livers was performed using the RNeasy kit (QIAGEN, Hilden, Germany), and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, NC). The reverse transcriptase reaction was done using random primers (Promega). The transcription

levels of mice collagen I (forward 5'TGACTGGAAGAGCGGAGAGT3' and reverse 5'ATCCATCGGTCATGCTCTCT3'), MMP-13 (forward 5' CCAGAACTTCCCAACCATGT3' and reverse 5'GTCTTCCCCGTGTTCTCAAA3'), and TIMP-1 (forward 5'ATCAGTGCCTGCAGCTTCTT3' and reverse 5'TGACGGCTCTGGTAGTCCTC3') were detected by quantitative real-time PCR methods with SensiMix™ SYBR kit (Bioline) on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA).

Quantification of data was performed via comparative ΔΔ Ct calculation (β-actin as housekeeping gene), and the gene expression levels in livers from CCL4- treated animals were set at baseline.

Western blotting. Proteins were isolated from frozen tissue (30-40 mg) of each animal. Samples (100 μg) of each lysate were separated in a 10% sodium dodecyl SDS gel. After that, the proteins were transferred to a PVDF membrane, which was blocked in 5% blotting grade blocker non-fat dry milk (BIO-RAD, 170-6404) for 2 h. β-actin (Sigma, A5316, 1:5000) was used as a control for equal loading of protein samples. Primary antibodies were diluted and added as follows: Collagen I (Southern Biotech, 1310-01, 1:250); MMP-13 (Santa Cruz, SC12363, 1:100); TIMP-1 (Santa Cruz, SC6834, 1:200); YM1 (R&D systems, AF4226, 1:200); IFR5 (Proteintech Europe, 10541-1-AP, 1: 800); and FOXP3 (eBiosciences, 13-5773-82, 1:100) and the membranes with the primary antibodies were incubated at 4°C overnight, then washed for 30 min with 0,1% Tween in TBS and incubated with secondary antibodies.

Computerized densitometry was used to measure the intensity of the bands and the relative intensity was determined through the ratio of the intensity of primary ab:β-actin (GeneSnap, SYNGENE).

Statistical analysis. Results were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni test. $P < 0.05$ was considered statistically significant.

Results

Modification, characterization and bioactivity of IL10-PEG-PPB. PPB was coupled to IL10 via 2KDa MAL-PEG-SCM as illustrated in fig. 1A and the conjugate was characterized by western blot, using anti-IL10 and anti-PPB antibodies (Fig. 1B). IL-10 showed a band at ± 37 kD, corresponding to its dimeric (active) form. A shift in molecular weight was found for IL10-PEG-PPB, with a major band between 50-70KDa, as shown after anti-IL10 and anti-PPB stainings. As chemical modification of IL-10 could affect its biological activity, anti-inflammatory effects of IL-10 and IL10-PEG-PPB were assessed in LPS-stimulated RAW 264.7 cells. When stimulated with LPS, RAW cells released

significantly more TNF-α compared to the control, as shown in fig. 2. This LPS-induced activation could be inhibited for 40±6,5% by IL-10 (***p<0.001) and 34±15% by IL10-PEG-PPB(***p<0.001) at a concentration of 50µg/mL for each compound. This indicates that IL-10 activity is preserved after modification of the molecule.

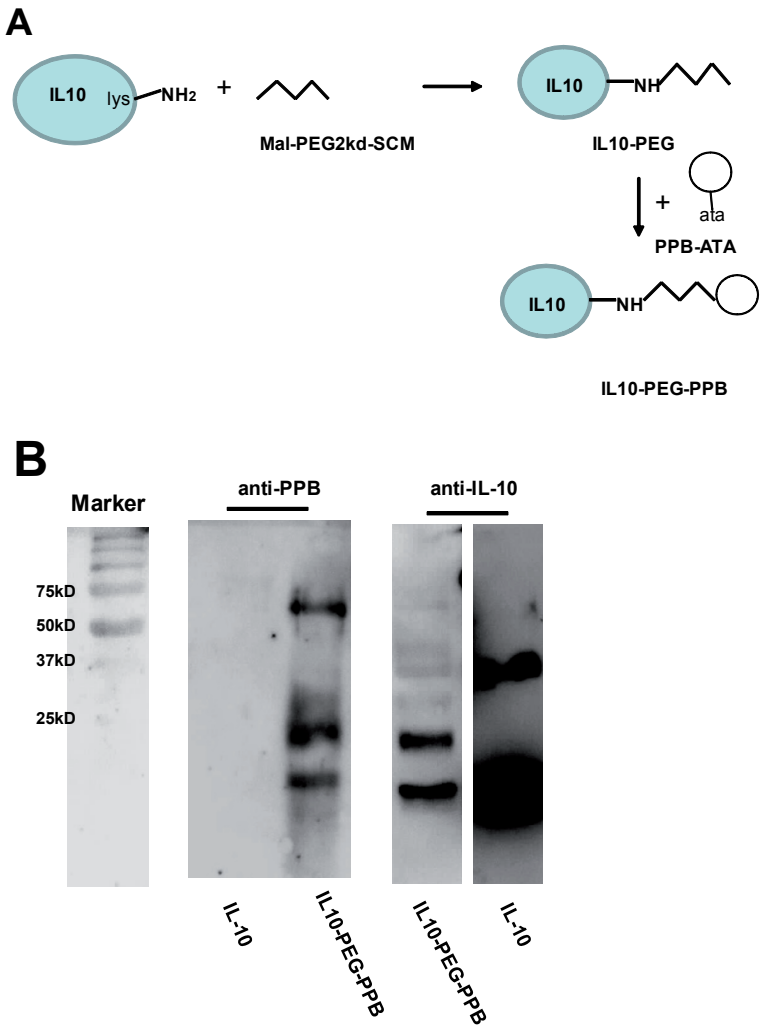

Fig. 1. *Preparation of IL10-PEG-PPB. (A) Schematic illustration of the synthesis of IL10-PEG-PPB. IL-10 was reacted with maleimide-PEG-succinimidyl carboxy methyl ester (MAL-PEG-SCM, 2kDa) and subsequently with PPB-ATA (B) Western blot analysis of native and modified IL-10 using anti PPB (left panel) and anti-IL-10 (right panel) antibodies. It can be seen that IL10-PEG-PPB stains positive for PPB and IL-10 and a shift in molecular weight of IL10-PEG-PPB can be observed after conjugation.*

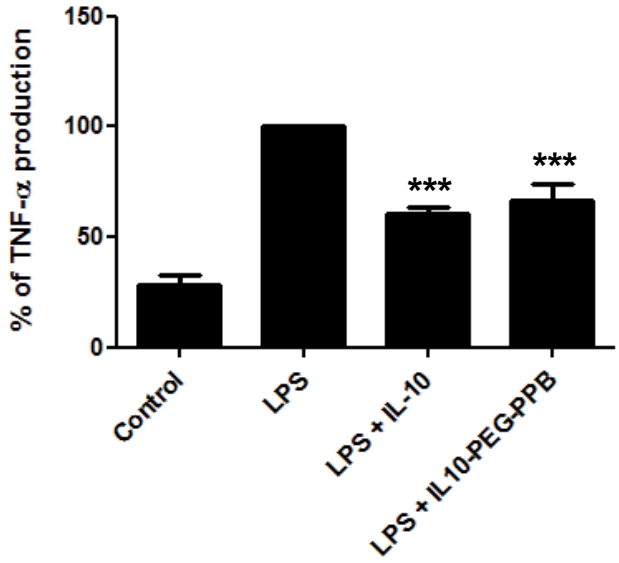

Fig. 2. *Effect of IL-10 and IL10-PEG-PPB on TNFα release by RAW 264.7 cells in vitro. Cells were incubated with 50 ng of IL-10 and IL10-PEG-PPB 30 min before a challenge with LPS (25 ng/mL). It can be seen that TNF-α production by RAW 264.7 cells is significantly inhibited by both IL-10 and IL10-PEG-PPB (***p< 0.001 compared to LPS-stimulated cells). Results are expressed as mean ± SD (n = 3).*

***In vivo* effects of IL10-PEG-PPB on collagen I.** To study the effects of IL10-PEG-PPB *in vivo*, we used the chronic CCl_4 mouse model. After 8 weeks of CCL_4 administration, deposition of fibrillar collagen (assessed by collagen type I expression) was extensive, as depicted in figure 3A and B. IL-10 treatment did not induce any significant effect but IL10-PEG-PPB significantly increased Collagen I deposition (*p<0.05). This was also seen after measurement of mRNA expression levels for collagen I (Figure 4A); only the group treated with the targeted IL-10 showed a significant upregulation (**p<0.01) as compared to IL-10 and the control group. Western blot analysis confirmed the increased collagen I deposition in the IL10-PEG-PPB-treated group as compared to the control and the IL-10 treated group (*p<0.05).

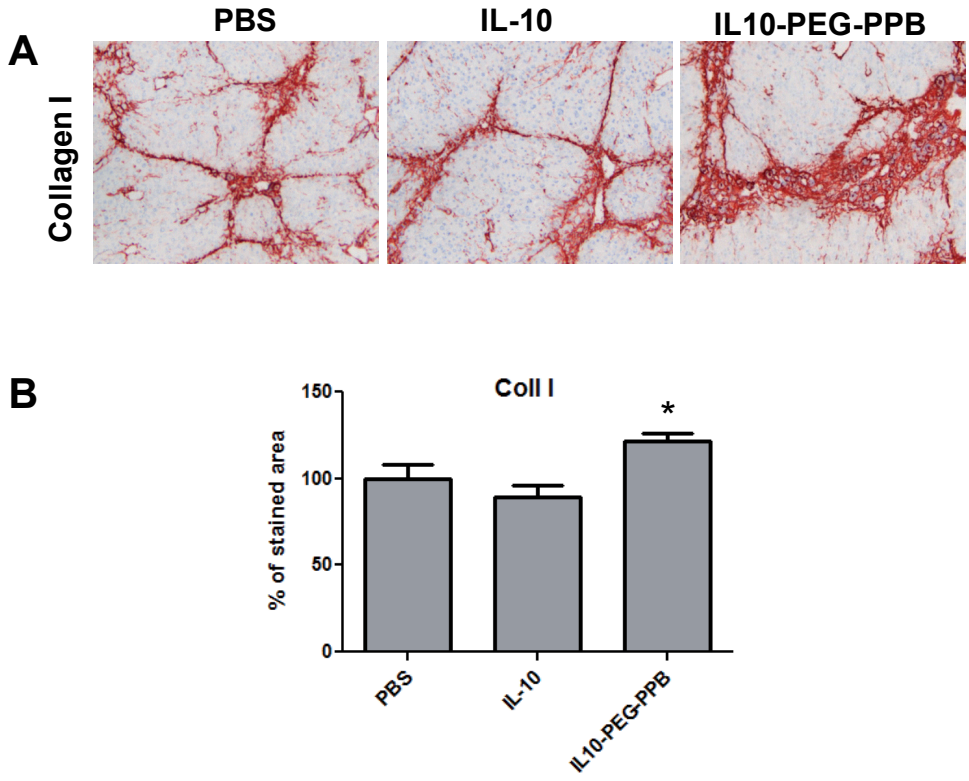

Fig. 3. *Effects of IL-10 and IL10-PEG-PPB in CCl4-induced liver fibrogenesis in mice. (A) Representative pictures of immunohistochemical staining for Collagen type I on liver cryostat sections. Animals received CCl4 injections for 8 weeks and were treated with PBS, IL-10 or IL10-PEG-PPB (dose 10 µg\kg\day) in the last week; (B) Quantification of the positive stained area for Collagen I using the Olympus Cell D analysis program. Results are shown as mean ±SD (n = 6 per group). *= p<0.05, as compared to the PBS-treated group (ANOVA).*

Effects of IL-10 and IL10-PEG-PPB on MMP-13 and TIMP-1 of CCl4 fibrotic livers. The levels of MMP-13 and TIMP-1, accessed by western blot and real time PCR, show that the IL-10-treated group displayed a significant upregulation of MMP-13 protein expression, as shown in figure 5A (*p<0.05). This was confirmed by RT-PCR (**p<0.01) (fig. 5C). TIMP-1, the inhibitor of MMP-13, was upregulated in the IL10-PEG-PPB-treated group at both protein and mRNA levels as compared to the control and to the IL-10 treated group, as shown in figures 5B and 5D (*p<0.05).

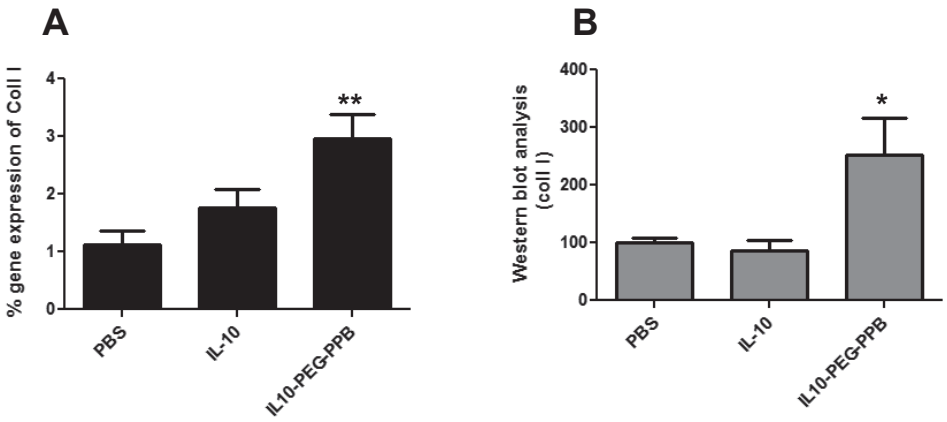

Fig. 4. *Effects of IL-10 and IL-10-PEG-PPB on the hepatic mRNA and protein levels for collagen I in vivo. (A) Collagen type I mRNA levels in CCl4-injected mice treated with PBS, IL-10 or IL10-PEG-PPB (n = 6 per group). ** = p<0.01compared to PBS and IL-10 treated mice. (B) Western blot analysis of collagen I deposition in CCl4-injected mice treated with PBS, IL-10 or IL10-PEG- PPB. * = p<0.05 compared to IL-10 or PBS-treated groups.*

Effects of IL-10 and IL10-PEG-PPB on macrophages of CCL4 fibrotic livers. Western blotting revealed a significant upregulation of the M2 marker YM1 (fig. 6A) and a downregulation of the M1 marker IRF5 (fig. 6B) in livers of the IL10-PEG-PPB treated group as compared to the untreated or the IL-10 treated group (*p<0.05). FOXP3, which is a marker for deactivated macrophages (M2c) and is known to be induced by IL-10, was significantly higher in both IL-10 and IL10-PEG-PPB treated groups compared to fibrotic livers of untreated mice, as shown in figure 6C (**p<0.01).

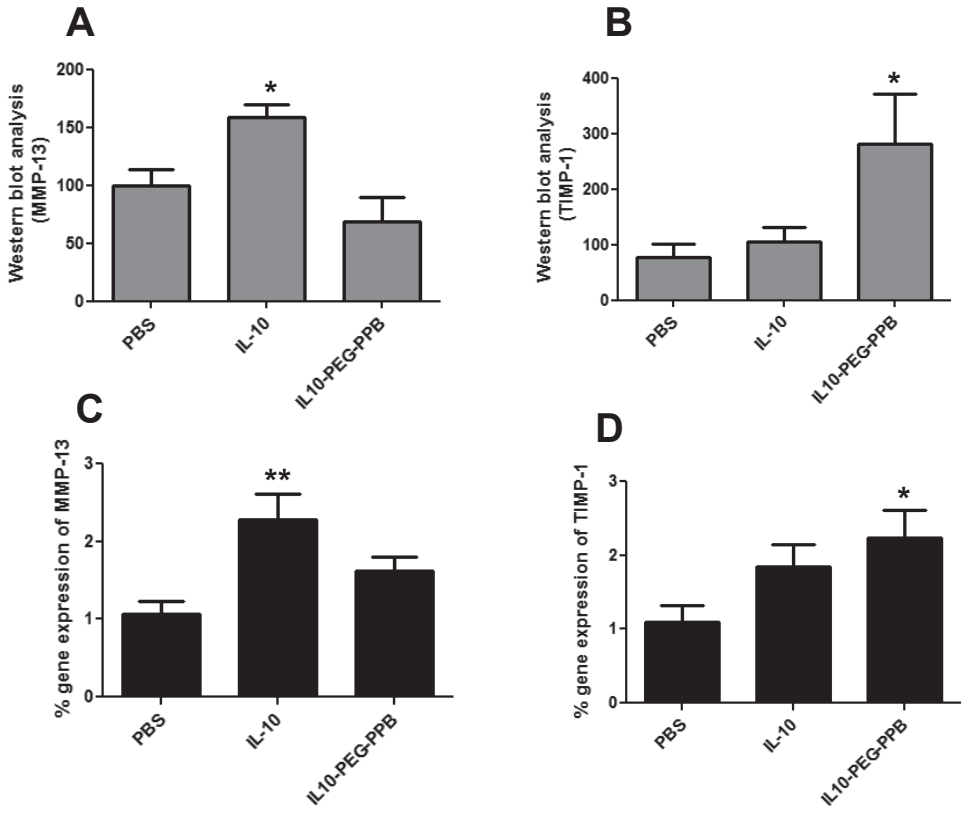

Fig. 5. *Effects of IL-10 and IL10-PEG-PPB on MMP-13 and TIMP-1 expression levels in vivo. (A) and (B) depict protein levels as measured by Western blot analysis. (C) and (D) show mRNA levels as measured by rtPCR. It can be seen that IL-10 significantly affects MMP-13 levels, whereas IL10-PEG-PPB significantly affects TIMP-1 levels. * = p<0.05 compared to PBS-treated fibrotic mice.*

Effects of IL-10 and IL10-PEG-PPB on MMP-13 and TIMP-1 expression in RAW 264.7 cells. To check whether the effect of IL-10 on macrophages could account for the observed changes in MMP-13 and TIMP-1 expressions, we stimulated RAW264.7 cells with IFNγ (to induce M1 polarization) or IL-13 (to induce M2 polarization) and subsequently cells were treated with IL-10. In figure 7A it can be seen that only M1 macrophages (i.e. IFNγ stimulated cells) displayed an increased expression of MMP-13 (***p<0.001) and after incubation with IL-10, these M1 polarized macrophages were even more active in transcripting MMP-13 (***p<0.001). On the other hand,

TIMP-1 expression was upregulated by IL-10 in both M1 and M2 macrophages. (***p<0.001). Collectively, our data show a shift from M1 to M2 macrophages induced by (modified) IL-10, both *in vitro* and *in vivo*. The phenotypical polarization was paralleled by changes in matrix metalloproteinases and M1/M2- associated cytokines both *in vitro* and *in vivo*. *In vivo*, this shift was most pronounced in animals treated with IL-10-PEG-PPB.

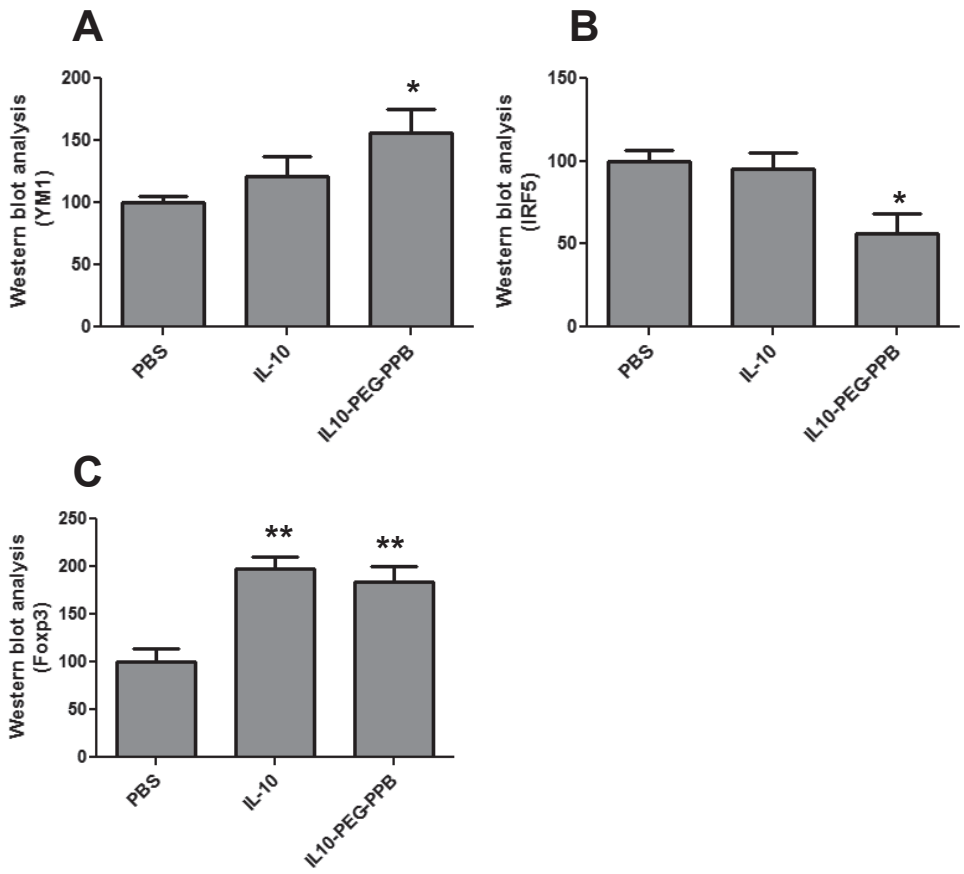

Fig. 6. *Western blot analysis of macrophage profiles in fibrotic livers treated with IL-10 or IL10-PEG-PPB. (A) Quantitative analysis of western blots for YM1 expression (M2 marker). *= p<0.05 compared to PBS-treated fibrotic mice. (B) Quantitative analysis of western blot for IRF5 expression (M1 marker) *= p<0.05 compared to IL-10 or PBS-treated groups. (C) Quantitative analysis for intrahepatic Foxp3 expression (marker for M2c- subtype). ** = p<0.01compared to PBS-treated group.*

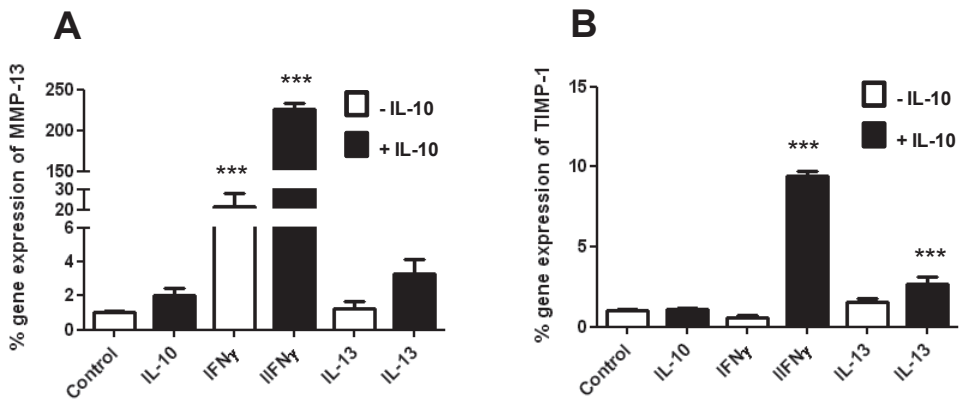

Fig. 7. *Effects of IL-10 on MMP-13 (A) and TIMP-1 (B) mRNA expression levels in RAW 264.7 cells. Cells were pre-incubated with IFNγ or IL-13 24 h before incubation with IL-10 to induce either an M1 phenotype (IFNγ pre-incubation) or an M2 phenotype (IL-13 pre-incubation). It can be seen that IL-10 exerts significant effects on MMP-13 expression only in cells pre-stimulated with IFNγ (M1-inducer), whereas its effects on TIMP-1 expression are most prominent in cells pre-stimulated with IL-13 (M2 inducer). ***= p<0.001 compared to control.*

Discussion

Interleukin-10 is a well-known immunoregulatory factor and was reported to be a potent anti-inflammatory cytokine that is able to decrease the expression of many pro-inflammatory cytokines [33]. Because of these anti-inflammatory effects, its relevance as an antifibrotic cytokine was considered [34] and indeed some studies found antifibrotic effects of IL-10, forming the basis for a clinical trial in fibrotic patients [35]. However, other studies revealed that IL-10 had pro-fibrotic effects [22,36]. As IL-10 has a short half-life in plasma and receptors on many cell types, this might explain the divergence in results and the targeting of IL-10 to HSC could be a suitable solution for this [37]. Activated HSC play a key role in fibrogenesis by producing an excess of ECM during the fibrotic process [2] and they are also the main source of TIMP-1 that is known to inhibit the activity of collagen degrading enzymes like MMP-13, thus shifting the balance towards fibrosis [19,20].

In previous studies, cytokines were modified to improve the pharmacokinetic or therapeutic profile [31,38,39]. IL-10 was previously modified with M6P (M6P-IL10) which induced an improvement of its pharmacokinetic profile with a remarkable antifibrotic activity *in vitro*, but there was still some uptake in Kupffer and

endothelial cells next to the accumulation in HSC [31]. In 2003, Beljaars et al synthesized PPB-albumin that was specifically delivered to the PDGFβ-receptor, which is highly upregulated on activated HSC during fibrogenesis [40]. Interferon γ (IFNγ) was modified with this PPB, via a PEG linker, and accumulated in activated HSC in a murine CCl4-induced fibrosis model where it significantly decreased fibrosis [39].

In the present study, we chemically modified IL-10 with PPB, via a PEG linker [39] to achieve high uptake in HSC and avoid uptake in immune cells. We hypothesized that the difference between HSC-targeted and native IL-10 *in vivo* might give information on the effect of IL-10 on different cell types. We confirmed that the biological activity of IL-10 was preserved after modification using an *in vitro* system to assess anti-inflammatory IL-10 properties [41] so we pursued our studies *in vivo* in a chronic model of liver fibrosis induced by CCl_4 in mice. During fibrogenesis, collagen deposition is increased and IL-10 has been reported to downregulate its expression [10,11,17], but in the present study IL-10 did not induce any significant decrease in collagen, although an upregulation of MMP-13 was found. This might be due to the short time of treatment. However, our results did show a significant increase in collagen deposition induced by IL10-PEG-PPB, which was paralleled by an upregulation of TIMP-1 in the IL10-PEG-PPB treated group and not in the mice treated with IL-10. So, IL-10 delivered to HSC is profibrotic and this effect seems to be at least in part due to an overexpression of TIMP-1.

HSC are a major source of TIMP-1 [42], whereas MMP13 is mainly produced by KC [43]. The increased expression of TIMP-1 in livers induced by IL10-PEG-PPB treatment suggests that IL10-PEG-PPB exerts its effects through modulation of HSC, whereas the increased expression of MMP-13 observed after IL-10 treatment indicates that native IL-10 exerts its effects via KC or macrophages. Activated HSC have a high expression of PDGFβ-receptor (the target for PPB) [40], which is absent on RAW macrophages and KC (data not shown). Delivery of IL-10 to the PDGFβ-receptor enabled us therefore to avoid macrophage uptake and favor HSC accumulation. Recent studies show that paracrine interactions between liver associated macrophages and HSC are important during fibrogenesis [44]. Our study supports this by showing that IL-10 delivered to HSC significantly affects polarization of macrophages by causing a shift from the M1 to the M2 phenotype. It can be envisioned that local production of IL-10 by hepatocytes, KC or HSC leads to a mixed response of HSC and KC. The net results will be dependent on the ratio between KC and HSC within the diseased area: during a local excess of KC, the antifibrotic effects of IL-10 will prevail, yet during a local excess of HSC, IL-10 will have pro-fibrotic effects via TIMP-1 upregulation. Our results indicate that these HSC subsequently promote M2 polarization, because an increase in intrahepatic M2 markers, paralleled by a decrease in M1 makers was found after delivery of IL-10 to the PDGF-receptor

on HSC. This supports the recent studies revealing the interaction between HSC and macrophages during fibrogenesis [44].

Several subpopulations of macrophages play different, sometimes even opposing roles during the process. Understanding of macrophage plasticity might be crucial to understand fibrotic mechanisms [33]. Therefore, we decided to analyze whether the targeting to HSC affected macrophage differentiation. IL-10 is known to promote alternative activation of macrophages (M2 phenotype), and also to downregulate its activities (M2c phenotype) [27]. Regulatory M2c macrophages provide anti-inflammatory effects [45], but their role in fibrosis is not well established. M2 macrophages are associated with pro-fibrogenic activities [46]. Treatment of mice with IL10-PEG-PPB induced a significant upregulation of the marker for M2 macrophages (YM-1) within livers, while the marker for M1 macrophages (IRF5) was downregulated. This is compatible with a shift of macrophages from an M1 to an (profibrotic) M2 profile. It corroborates the finding of an upregulation of collagen I and TIMP-1 levels in this group.

To confirm that different types of macrophages might play different roles during fibrogenesis and that IL-10 is able to induce the changes we observed *in vivo* we decided to conduct *in vitro* experiments in a mouse macrophage cell line (RAW 264.7). These studies confirm that IL-10 is able to induce anti-fibrotic activities in M1 macrophages, at least in part by inducing a release of MMP-13 (see fig. 5A and 7A). In M2 macrophages IL-10 stimulates TIMP-1 production, thus stimulating fibrogenesis.

In summary, when IL-10 was delivered to the HSC, thus avoiding macrophage binding, pro-fibrotic effects were found, associated with a polarization of macrophages to the M2 phenotype, a decreased MMP-13 and an increased TIMP-1 expression. In contrast, native, untargeted IL-10 induced a strong MMP-13 upregulation, most likely via an effect on KC/macrophages. Our studies do not exclude an additional effect of native, unmodified IL-10 via immunosuppression, because only the effect of IL-10 on liver associated macrophages and HSC was examined here.

Collectively, our study indicates cross talk between HSC and macrophages that affects cell differentiation, with an important regulatory role for IL-10 in this process. The different, sometimes opposing effects of IL-10 on macrophages and HSC also explains many of the contradictory effects of IL-10 on fibrogenesis seen in other studies [35][23,47-49]. Further studies are necessary to elucidate this pathway.

Aknowledgements

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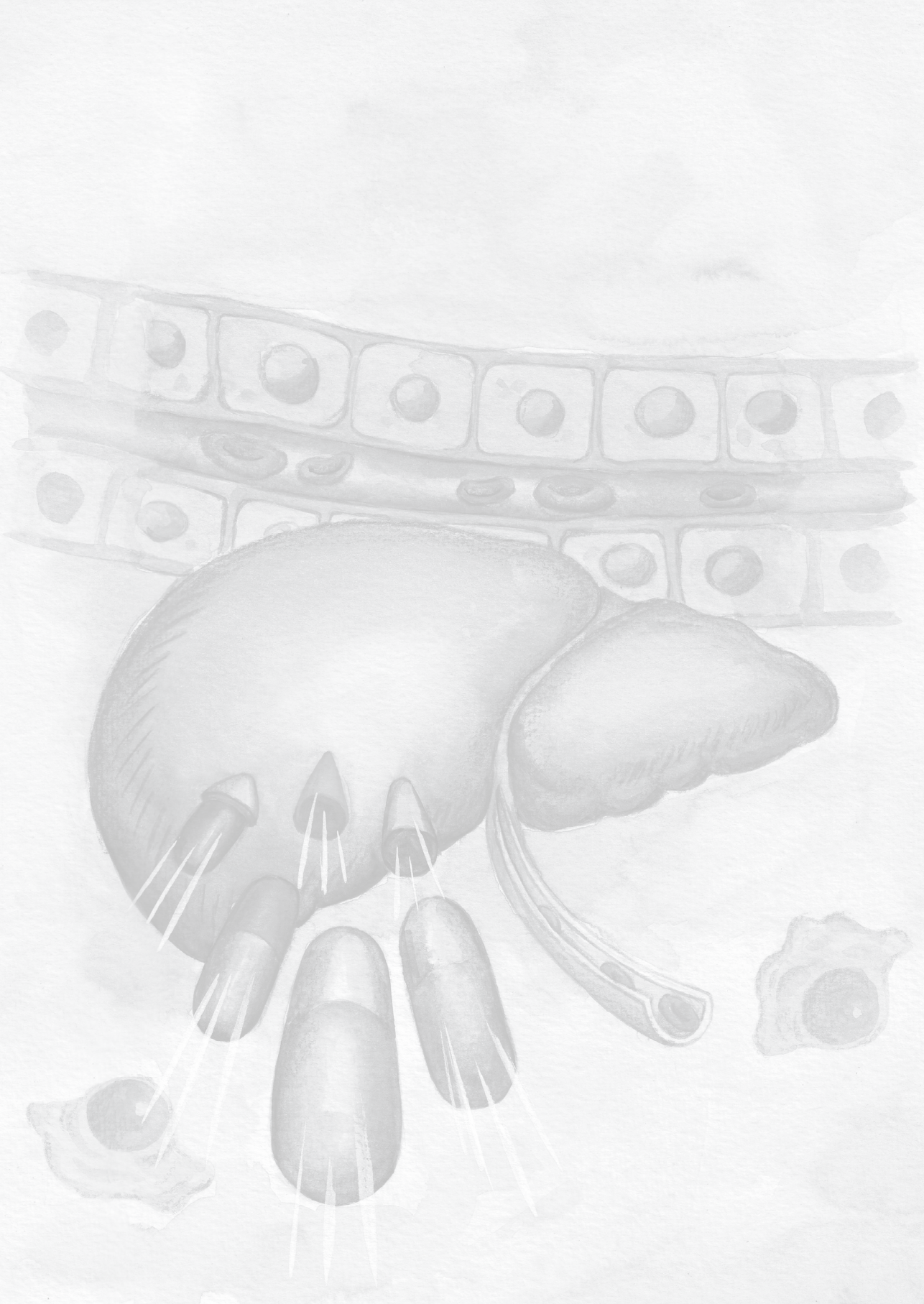

Chapter 5

Interleukin-10 steers the hepatic stellate cell- macrophage axis during liver fibrogenesis

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Article submitted

Abstract

The immunoregulatory and anti-inflammatory Interleukin-10 (IL-10) was found to have antifibrotic effects in some studies yet profibrotic effects in other. This might be due to different effects on the immune system and tissue-resident cells. To unravel the exact role of IL-10 during liver fibrogenesis we delivered this locally acting cytokine to activated hepatic stellate cells (HSC) in a chronic model of CCl4-induced liver fibrosis. HSC are key cells that control fibrogenic activity. We coupled a cyclic peptide (PPB) that binds to the PDGF-β-receptor, which is abundantly expressed on activated HSC, to IL-10 via a PEG-linker, to enhance stability. Balb/c mice receiving CCl_4 for 8 weeks were treated for 1 week with this construct (IL10-PEG-PPB), unmodified IL-10 or vehicle (n = 6 per group). The present results reveal an increased deposition of collagen and fibronectin in the group that was treated with IL10-PEG-PPB as compared to untreated or IL-10-treated animals. The number of activated HSC was not affected by IL-10 or IL10-PEG-PPB, but the marker for Kupffer cells F4/80 was significantly upregulated in IL10-PEG-PPB-treated mice. This was associated with a significant decrease in MCH class II expression levels and upregulation of TGFβ production in this group. In addition, increased expression levels of the CCR2 receptor and its ligand CCL2 were found after administration of IL-10 or IL10-PEG-PPB. So, delivery of IL-10 to activated HSC led to an increased expression of the macrophage chemotactic CCL2/CCR-axis. This was associated with an enhanced macrophage influx, yet reduced MHC class II expression. Our results therefore indicate that IL10-PEG-PPB stimulates the influx of macrophages of the M2c subtype, endowed with profibrotic activities. This study reveals the local role of IL-10 in HSC-macrophage interactions during fibrogenesis.

Keywords: *Interleukin-10, drug targeting, hepatic stellate cells, liver fibrosis, macrophages, CCR2/CCL2.*

Introduction

Liver fibrosis is commonly associated with chronic inflammation but most treatments for liver fibrosis and other fibrotic disorders based on blocking inflammatory processes have not been effective. One of the reasons for this could be that the mechanisms that are involved in fibrogenesis are distinct from those involved in inflammation [1].

Hepatic fibrogenesis represents a leading cause of morbidity and mortality worldwide and is the result of a complex interplay between liver cells. Particularly activated hepatic stellate cells (HSC) play a central role in mediating actions of resident hepatic cell types during fibrogenesis. In a healthy liver, quiescent HSC store vitamin A, but upon activation this cell type acquires a fibroblast-like phenotype, starts to proliferate and becomes the main source of extracellular matrix proteins, especially collagen type I and type III [2,3]. In addition, activated HSC secrete pro-fibrotic and pro-inflammatory mediators that perpetuate disease activity and attract immune cells from the blood into the injured liver, including macrophages/monocytes [4,5]. In recent years, macrophages gained more attention for their role in fibrogenesis [6-9]. Their polarization into different subtypes appears to be important in both resolution and exacerbation of fibrosis [10,11]. Macrophages hold important roles in a variety of physiological and pathological processes, such as host defense, acute and chronic inflammation, and tissue remodeling. They can be phenotypically polarized into M1 (classically activated macrophages, activated by IFN-γ and LPS) which drive pro-inflammatory responses, or M2 (alternatively activated macrophages) which are responsible for immunoregulation and/or tissue remodelling [11-13].

Cytokines regulate communication between different liver cells during fibrogenesis. An important member of the cytokine family is IL-10 which is produced by various cell types, including T and B cells, keratinocytes and macrophages [14]. Within the liver, resident macrophages, hepatocytes and hepatic stellate cells are all known to produce IL-10 [15]. The ability of IL-10 to inhibit inflammatory responses led to the hypothesis that IL-10 could be involved in the resolution of fibrosis [16]. Indeed some studies revealed an anti-fibrotic effect of IL-10 in several species, including humans [17-20]. One of the mechanisms of IL-10 in the resolution of liver fibrosis may be related to the up-regulation of matrix metalloproteinase-13 (MMP-13) in macrophages and the inhibition of tissue inhibitor of metalloproteinase-1 (TIMP-1) in HSC [21-24], both key players during removal of the matrix. However, many studies found pro-fibrotic effects of IL-10 during skin, renal and pulmonary fibrosis [25-27]. Recently, we also found that IL-10, re-directed to HSC, induced profibrotic effects, accompanied by up-regulation of gene expression for TIMP-1 (Chapter 4). We hypothesized that, by specifically targeting IL-10 to HSC, uptake in macrophages or other immune cells was inhibited, preventing its pleiotropic effects on the immune system. The different effects of IL-10 on the immune system and tissue-resident cells might account for the opposite effects of this cytokine seen in several studies.

In the present study, we aim to further elucidate the complex role of IL-10 in the interaction between HSCs and liver macrophages. For this purpose IL-10 was chemically modified with PPB, the cyclic peptide that binds to the PDGF-β-receptor, to specifically target it to HSC [28,29]. We explored its effects on fibrogenesis and macrophage activities during CCl_4-induced liver fibrosis in mice. Our present results show that IL-10 delivered to HSC enhances fibrosis and affects the polarization of macrophages. The CCL2/CCR2 axis appears to play an important role in this communication between HSC and macrophages. This study on IL-10 provides new insights into the communication between hepatic cells during liver fibrosis.

Material and methods

IL10-PEG-PPB synthesis, characterization and activity. The conjugation and characterization of PPB to IL-10 was done according to a similar procedure as for Interferon γ [30]. Briefly, 50 μg/mL of human IL-10 (rhIL-10, PeproTech EC Ltda., UK) was reacted for 2 h, in PBS buffer, with maleimide-PEG-succinimidyl carboxy methyl ester (MAL-PEG-SCM, 2kDa, Creative PEGworks, Winston Salem, NC). After dialysis, the product was incubated with PPB-ATA (ON, 4°C). The purified conjugate IL10-PEG-PPB was subsequently dialyzed against PBS for 24 h and stored at -20°C. Western blot using anti-IL10 and anti-PPB antibodies were used to confirm the conjugation. Biological activity of IL10-PEG-PPB was verified in RAW 264.7 cells (ATCC, TIB-71) by measuring the effect of compounds on the LPS-induced TNFα-response.

Animals and experimental model of liver fibrosis. Male BALB/c mice for animal experiments were purchased from Harlan (Zeist, the Netherlands) and kept at laboratory conditions with *ad libidum* chow and water. The Animal Ethics Committee of the University of Groningen, the Netherlands, approved all the experimental procedures described here. Chronic liver fibrosis was induced by intraperitoneal injections of CCl_4 (0.5 mL/Kg in the first week; 0.8 mL/Kg in the second week and 1 mL/kg in the remaining weeks, all in olive oil). To study the effect of our compounds, animals received daily an i.v. injection of PBS, IL-10 or IL10-PEG-PPB (10 μg/Kg/day) in the last week of the CCl_4-treatment. Animals were sacrificed 24 h after the last CCl_4 injection.

Immunohistochemistry. Assessment of expression of type III collagen and MHC class II was done in 4μm cryostat liver sections fixated in acetone and stained with indirect immunoperoxidase methods. Briefly, sections were incubated for 1 h with goat anti-collagen III (Southern Biotech, USA) or rat anti-MCH class II (Santa Cruz

Biotechnology, USA) IgGs. Suitable secondary and third antibodies were applied and visualized with 3-amino-9-ethylcarbazole (AEC, Sigma), according to standard methods. Staining of MHC class II was quantified by image analyzing techniques, using Multiple Alignment Analysis (MIAs). The results were given as percentages of positive-stained areas divided by the total area of the section.

Real-Time PCR. Isolation of total RNA from mice livers was performed using the RNeasy kit (QIAGEN, Hilden, Germany), and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, NC). The reverse transcriptase reaction was done using random primers (Promega). The transcription levels of mice collagen III (forward 5'ACGTGGTAGTCCTGGTGGTC3' and reverse 5'GCTGGTCCAGCATCACCTTTT3'), α-SMA (forward 5'ACTACTGCCGAGCGTCAGAT3' and reverse 5'CCAAATGAAAGATGGCTGGAA3'), F4/80 (forward 5'TGCAATCTAGCAATGGACAGC3' and reverse 5'GCCTTCTGGATCCATTTGAA3'), CCR2 (forward 5'AGGGCATTGGATTCACCACAT3' and reverse 5'TACGGTGTGGTGGCCCCTTCA3'), and IL-10 (forward 5'ATAACTGCACCCACTTCCCAGTC3' and reverse 5'CCCAAGTAACCCTTAAAGTCCTGC3') were detected by quantitative real-time PCR methods with SensiMix™ SYBR kit (Bioline) on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA). Quantification of data was performed via comparative ΔΔCt calculation (β-actin as housekeeping gene), and the gene expression levels in livers from CCl_4-treated animals and injected with PBS were set at baseline.

Western blotting. Proteins were isolated from frozen tissue (30-40 mg) of each animal. Samples (100 μg) of each lysate were separated in a 10% SDS gel. After that, the proteins were transferred to a PVDF membrane, which was blocked in 5% blotting grade blocker non-fat dry milk (BIO-RAD, 170-6404) for 2 h. β-actin (Sigma, A5316, 1:5000) was used as a control for equal loading of protein samples. Primary antibodies were diluted and added as follows: Fibronectin (Sigma, F6140, 1:500); α-SMA (Sigma, A2547, 1:1000); and CCL_2 (Santa Cruz, SC6834, 1:200) and the membranes with the primary antibodies were incubated at 4°C overnight, then washed for 30 min with 0,1% Tween in TBS and incubated with suitable secondary antibodies.

Computerized densitometry was used to measure the intensity of the bands and the relative intensity was determined through the ratio of the intensity of primary ab:β-actin (GeneSnap, SYNGENE).

Statistical analysis. Results were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni test. $P< 0.05$ was considered statistically significant.

Results

The composition of the synthesized construct (IL10-PEG-PPB) was analyzed by checking the presence of the 3 different moieties in the construct. Western blots using an antibody against IL-10 revealed a band at ± 37 kD for native IL-10 and a major band between 50-70kD for IL10-PEG-PPB (data not shown). Immunoblotting with antibodies against PEG and PPB further confirmed the presence of both moieties in our construct (data not shown). Bioactivity of IL10-PEG-PPB was subsequently assessed in bioassays in cultures of RAW 264.7 Cells; IL10-PEG-PPB was found to attenuate the LPS-induced TNFα responses in these cells to a similar extend as native IL-10: 40±6.5% reduction in TNFα levels for IL-10 and 34±15% reduction for IL10-PEG-PPB compared to untreated RAW cells (***p<0.001 in both cases).

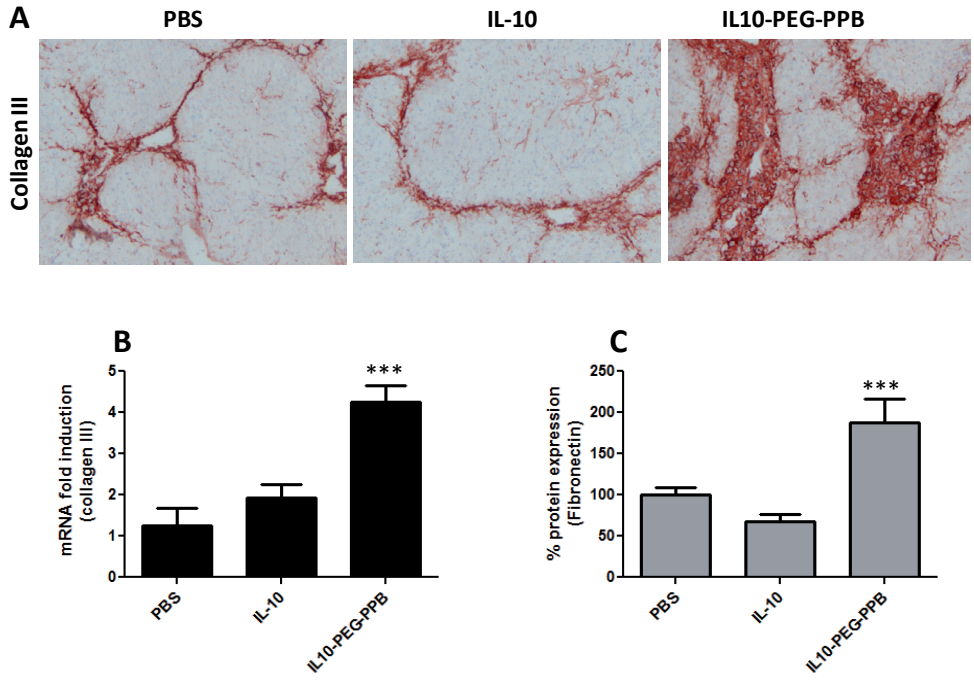

Fig. 1. ***Effects of IL-10 and IL10-PEG-PPB in CCl4-induced liver fibrogenesis in mice (n = 6 per group).*** *Animals received CCL4 injections for 8 weeks and were treated with PBS, IL-10 or IL10-PEG-PPB (dose 10 μg\kg\day) in the last week. (A) Representative pictures of immunohistochemical staining for Collagen type III on liver cryostat sections. (B) mRNA levels for collagen type III in the different experimental groups (***p<0.001 compared to PBS and IL-10 treated animals). (C) Western blot analysis of fibronectin protein expression levels; *** = p<0.001 in the IL10-PEG-PPB treated group as compared to the untreated and IL-10 treated groups.*

Effects of IL10-PEG-PPB on fibrogenesis in vivo. To study the effects of IL10-PEG-PPB on fibrosis *in vivo*, we used a chronic CCl_4 model in mice. After 8 weeks of CCL_4 administration, deposition of fibrillar collagen III was extensive, as shown by immunohistochemistry (figure 1A) and mRNA expression levels (figure 1B). IL-10 treatment did not induce any significant effect but IL10-PEG-PPB significantly increased Collagen III mRNA expression (***p<0.001) compared to untreated fibrotic mice. Also fibronectin levels, representing another constituent of the extracellular matrix, were significantly increased in IL10-PEG-PPB-treated fibrotic mice, compared to mice receiving vehicle after CCl_4 administrations (Fig 1C). (***p<0.001).

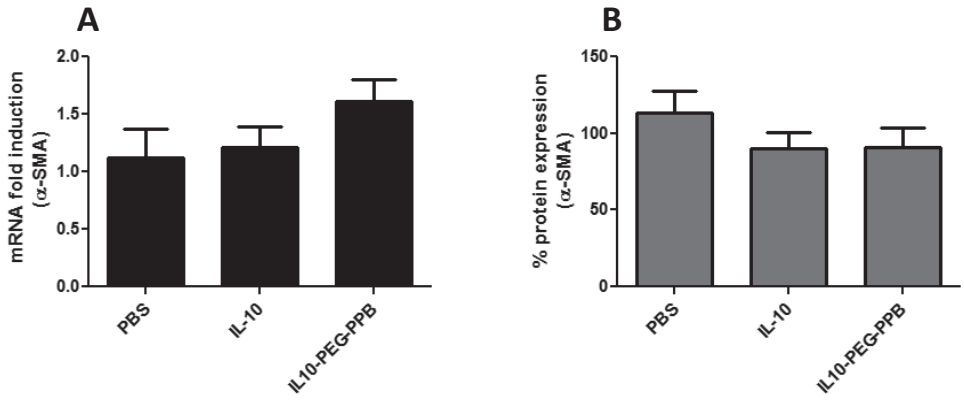

Fig. 2. ***Effects of IL-10 and IL-10-PEG-PPB on activated hepatic stellate cells (HSC).*** *(A) α-SMA mRNA expression levels and (B) western blot analysis of α-SMA in fibrotic mice receiving vehicle (PBS), native Il-10 or modified IL-10 (IL10-PEG-PPB). No significant differences were observed between groups.*

Effects of IL-10 and IL10-PEG-PPB on HSC activation. One of the key features of liver fibrosis is the activation of HSCs, that are characterized by an increased expression of α-SMA. However, both mRNA and protein expressions levels for α-SMA were not affected by neither IL-10 nor IL10-PEG-PPB in the present study (figures 2A and 2B, respectively).

Effects of IL10 and IL10-PEG-PPB on the recruitment of macrophages into fibrotic livers. The total number of macrophages was accessed by real time PCR using the marker F4/80 (Fig. 3). In the group treated with IL10-PEG-PPB, mRNA levels for F4/80 were significantly upregulated as compared to untreated animals or animals

receiving native IL-10 (*p<0.05). The lack of effect of the HSC-specific construct on HSC, combined with the increased F4/80 expression prompted us to examine the CCR2/CCL2 axis which plays a role in the communication between these cells [31]. There was a significant upregulation of CCR2 mRNA levels (fig. 4A) in both IL-10 and IL10-PEG-PPB-treated groups (*p<0.05 and **p<0.01 respectively). CCL2/MCP-1, a known promoter of macrophage infiltration and ligand for CCR2, was also upregulated in both groups (Fig. 4B; **p<0.01 for IL-10 and ***p<0.001 for IL10-PEG-PPB) as compared to the control.

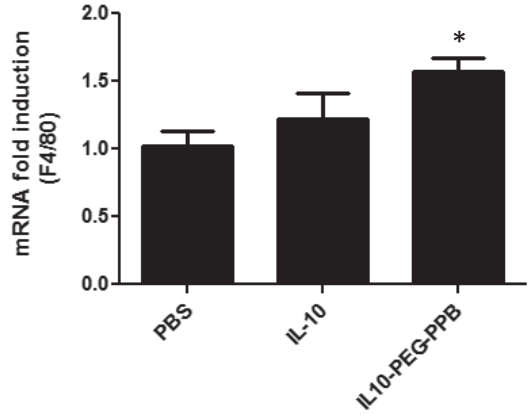

Fig. 3. ***Effects of IL-10 and IL10-PEG-PPB on number of macrophages****. Real time PCR showing significant increase in gene expression for F4/80 in the IL10-PEG-PPB-treated group as compared to animals treated with IL-10 or the untreated group. (*= p<0.05).*

Effects of IL-10 and IL10-PEG-PPB on MHC class II, IL-10 and TGF*β *in fibrotic livers. We used MHC class II, IL-10 and TGFβ expression levels to examine which cell-types respond to our treatments. IL-10 treatment of fibrotic mice did not induce a significant reduction in MHC Class II expression compared to untreated fibrotic mice (Fig 5A and 5B). However, the IL10-PEG-PPB treated fibrotic group showed a significant downregulation of MHC class II staining compared to untreated fibrotic mice (* p<0.05, Fig 5B). IL-10 mRNA expression levels in fibrotic livers were significantly upregulated in the IL-10 treated group (*p<0.05, Fig 5C) compared to untreated, whereas IL10-PEG-PPB did not have this effect on IL-10 mRNA levels. Treatment with IL10-PEG-PPB enhanced mRNA expression levels for TGFβ significantly (Fig 5D; *p<0.05), whereas the effect of native IL-10 was not significant.

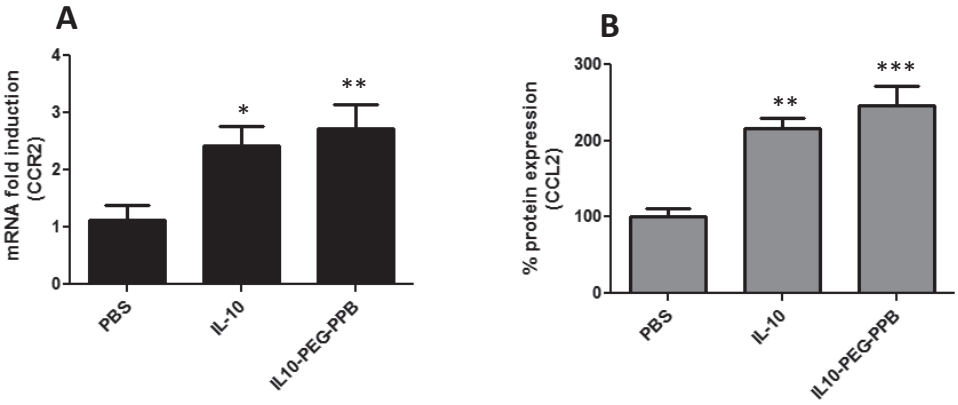

Fig. 4. ***Effects of IL-10 and IL10-PEG-PPB on CCR2 and CCL2 levels.*** *(A) Expression levels of mRNA for CCR2 in fibrotic mice receiving different treatments. *= p<0.05 and **= p<0.01 as compared to vehicle-(PBS)-treated group; (B) Western blot analysis of CCL2 protein levels in the different experimental groups. **= p<0.01 and ***= p<0.001 as compared to PBS group.*

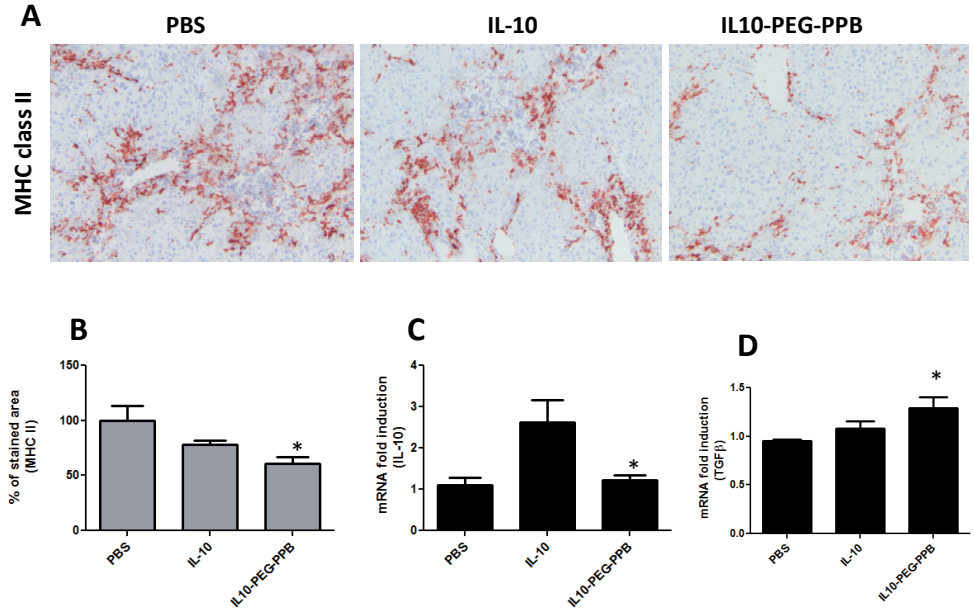

Fig. 5. ***Effects of IL-10 and IL10-PEG-PPB on MHC II, IL-10 and TGFβ on fibrotic livers.*** *(A) Representative pictures of immunohistochemical staining for MHC class II on liver cryostat sections; (B) Quantification of the positive stained area for MHC class II using the Olympus Cell D analysis program. *= p<0.05 as compared to untreated and IL-10 treated groups; (C) mRNA expression levels of IL-10. *= p<0.05 as compared to the PBS and to IL0-PEG-PPB-treated groups; (D) Gene expression levels for TGFβ in different experimental groups. *= p<0.05 as compared to untreated and IL-10-treated groups. Results are shown as mean ±SD (n = 6 per group).*

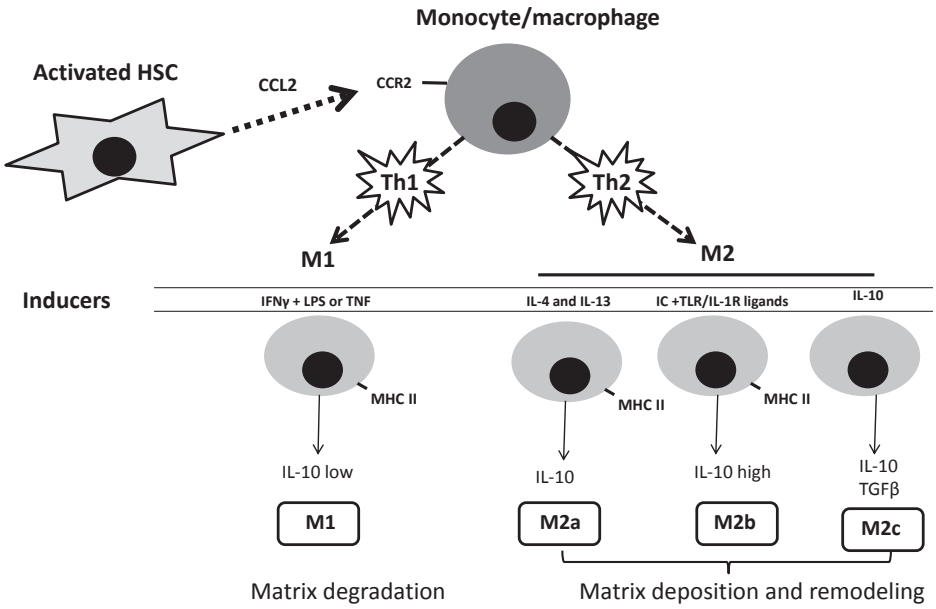

Fig. 6. *Schematic overview of the proposed HSC-macrophage interaction and an overview of the markers for the different subtypes of macrophages used in the present study. The combination of MHC class II, TGFβ and Il-10 expression can be used to identity a subset of macrophage (see also ref 59).*

Discussion

The role of IL-10 in inhibiting inflammatory responses led to the hypothesis that IL-10 could also be involved in the resolution of the fibrotic process [16], but its effects on fibrosis were limited or even profibrotic [32]. The most noticeable effect of IL-10 seems to be in immunomodulation with a broad range of anti-inflammatory effects [33-35]. IL-10 is reported to attenuate proinflammatory cytokine production [33] leading to therapeutic effects in some inflammatory diseases such as psoriasis and Crohn's disease [36-39]. Significant anti-fibrotic effects were found in some studies [40-42], but the exact role of IL-10 in fibrotic disorders remained unclear. In 2003, a clinical trial on the effects of IL-10 in patients with liver fibrosis was withdrawn because of the lack of efficacy and the adverse effects related to immune suppression [40]. There are many conflicting data on the fibrogenic effects of IL-10 as in several studies pro-fibrotic effects of this cytokine were observed in organs like skin, kidneys and lungs [25-27,43]. Recently we found that PEGylation of IL-10, causing a prolonged bioavailability of IL-10 *in vivo*, attenuated hepatic fibrosis and intrahepatic

inflammation in the CCl_4 model in mice associated with inhibition of HSC activation. Whether this antifibrotic effect of PEG-IL10 was a direct effect on HSC or secondary to its anti-inflammatory properties remained to be established.

To unravel the local role of IL-10 we now tested the effects of IL-10 directly delivered to the HSC. During hepatic fibrogenesis activated HSC display an upregulation of the PDGFβ-receptor [44-46]. A cyclic peptide that specifically binds to the PDGFβ-receptor (PPB) has previously been applied to deliver biologicals to activated HSC in fibrotic livers [46-48]. We used this peptide here to deliver IL-10 to HSC. Using the same approach presented here, our group recently demonstrated an increased collagen deposition after IL10-PEG-PPB administration, associated with an enhanced accumulation of M2 macrophages compared to animals treated with native IL-10 (submitted paper). Our present data confirm the profibrotic effects of the conjugate showing increased accumulation of ECM components after IL10-PEG-PPB administration. Surprisingly, neither the conjugate nor the native IL-10 had an effect on α-SMA expression, the marker for activated HCS (fig. 2A and 2B), although IL-10 was directed to this cell type. We neither did see an increased apoptosis of HSC, as reported in other studies [49] but this may be caused by the short-term IL-10 treatment in the present experiments. However, significant effects were found on the macrophage population. Targeting of IL-10 to the HSCs induced a significant increase in the number of Kupffer cells (fig. 3) as reflected by the upregulation of its marker F4/80 [50]. *In vitro* studies recently have shown that activated rat HSCs are able to induce polarization, at least *in vitro*, of macrophages [51] and we therefore hypothesized that IL-10 may affect HSC by inducing factors that subsequently affect macrophages.

There is accumulating evidence that macrophage polarization plays an important role in the pathogenesis of liver fibrosis. Classically activated macrophages (M1) exhibit proinflammatory and antifibrotic properties whereas alternatively activated macrophages (M2) are endowed with anti-inflammatory and profibrotic properties. M2 macrophages are part of a diverse population and so far 3 subtypes of M2 macrophages were identified: M2a (activated by IL-13 and IL-4), M2b (activated by immune complexes combined with IL1-R or TLR agonists), and M2c (activated by IL-10, TGFβ or glucocorticoids) [52-55]. Recent studies showed that HSC are able to affect macrophages by production of the chemotactic factor CCL2 [56,57], reason for us to examine CCL2 production and expression of its receptor CCR2. Our results show a significant increased expression of CCL2 and CCR2 mRNA levels in livers of fibrotic mice treated with IL-10 or its modified form IL10-PEG-PPB.

The CCL2/CCR2-axis is an important factor involved in the perpetuation of hepatic inflammation and fibrosis. The chemokine CC-chemokine ligand 2 (CCL2 in

mice or MCP-1 in rats and humans) is a potent chemotactic attractant for monocytes, macrophages and fibrocytes and other cell types with its cognate receptor (CCR2) [5]. CCL2 is known to have synergistic effects with pro-fibrotic cytokines (such as TGFβ) during the development of fibrosis [1,56,58]. In 2011, overexpression of IL-10 was found to be the cause of lung fibrosis and responsible for the recruitment of fibrocytes, via the induction of the CCL2/CCR2 axis [26]. In our study, we also found an upregulation of both CCR2 gene expression and CCL2 protein expression (fig. 4) induced by IL-10 and by IL10-PEG-PPB. This suggests that the increased influx of F4/80 positive cells (in IL10-PEG-PPB-treated animals significant compared to untreated fibrotic animals) is induced by activation of the CCL2/CCR2 axis by both compounds. In the case of IL10-PEG-PPB treatment this was associated with a significant enhancement of the fibrogenic process. However, this was not paralleled by a change in the number of activated HSC (fig 2), reason for us to examine the macrophage profiles in the different experimental groups.

Despite of the higher number of F4/80 positive Kupffer cells, a significant down-regulation of MHC class II expression in animals that received IL10-PEG-PPB was found. IL-10 is known to induce macrophage polarization into the M2c phenotype. These particular macrophages are characterized by low MHC class II expression and high TGFβ and IL-10 expression levels (see figure 6) [59][60,61]. Although in the IL10-PEG-PPB group, IL-10 mRNA levels were not increased, TGFβ levels were significantly higher in this group. TGFβ may be produced by M2c cells or by HSC, but in both cases it explains the increased fibrogenic response seen after local delivery of IL-10 to HSC. It is hypothesized that the mild and mixed responses seen after administration of native IL-10 is induced by a combination of local effects (leading to enhanced fibrogenesis) and systemic immunosuppressive effects (leading to reduced fibrogenesis). This can also explain the conflicting results obtained with IL-10 in different disease models, different tissues and different patient groups [40,62-64] .

In summary, the delivery of IL-10 to HSC using the construct IL10-PEG-PPB, significantly affected activities of resident hepatic cells. An increased accumulation of KC was found corresponding with an increased production of CCL2 and CCR2 receptor expression. The mediator profile found in IL10-PEG-PPB-treated mice suggests that this compound induced polarization of KC into the M2c subtype. This subtype exerts profibrotic effects, which might account for the increased deposition in ECM seen in these livers. Insight into the interaction between macrophages and HSC as obtained after local delivery of this cytokine demonstrates an important role for IL-10 in the cell-cell communication. In particular, unraveling the signals that affect the polarization of KC in pro-fibrotic or anti-fibrotic subtypes [8] may lead to new targets for the treatment of liver fibrosis.

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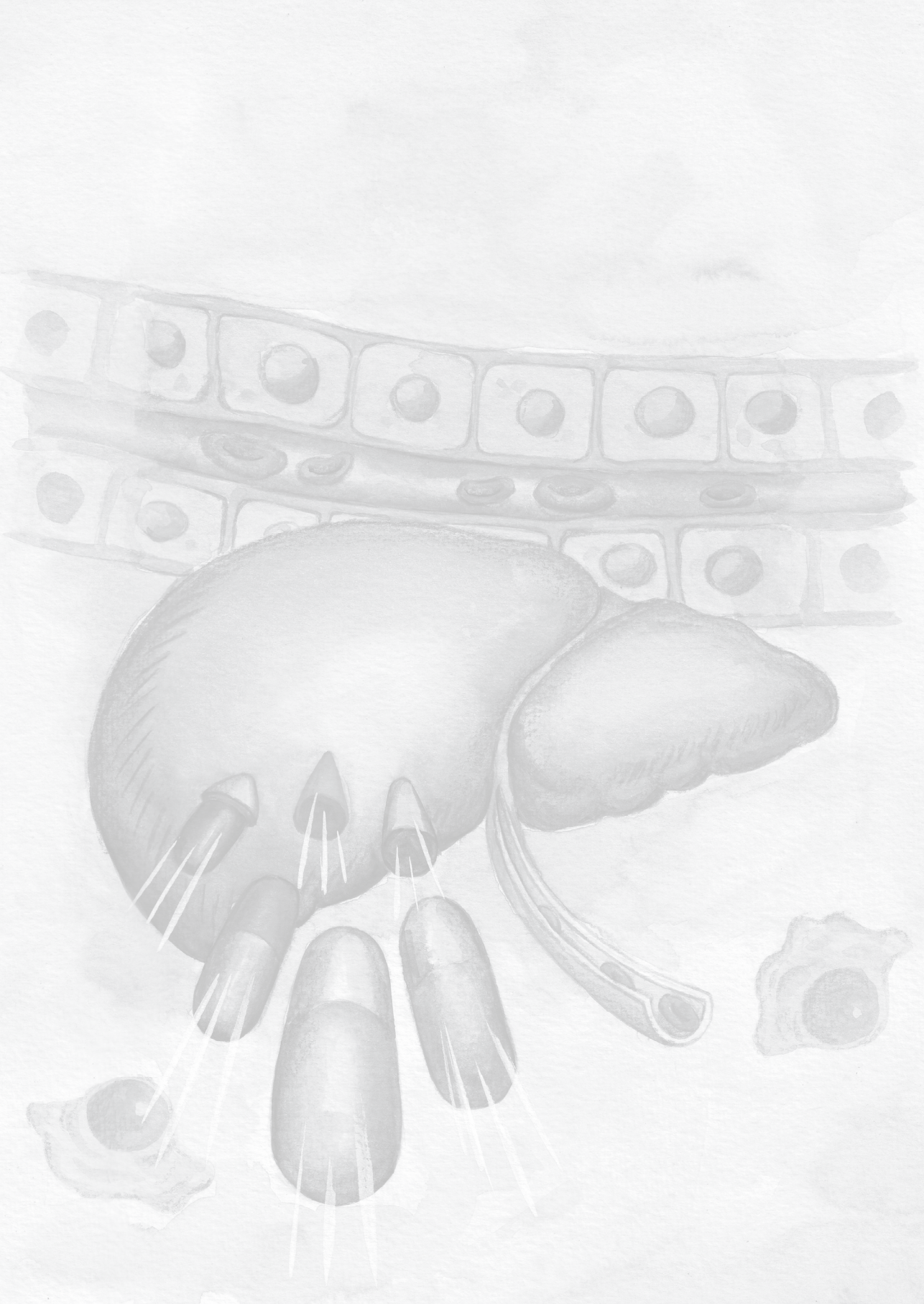

Chapter 6

Summary, general discussion and future perspectives

Summary and discussion

In this thesis we have focused on the cell-specific delivery of interleukin-10 (IL-10) to the activated Hepatic Stellate Cell (HSC) during liver fibrogenesis in mice. Up to now, removing the primary cause of liver fibrosis is known to stop the progression or even promote the reversal of the disease. However, this does not mean a total recovery and currently there is no therapy available to treat hepatic fibrosis, so there is a need for novel developments in this area.

Liver fibrosis is a significant health problem worldwide and the most common causes of its progression are chronic hepatitis C and B, alcohol or drug abuse and genetic or metabolic disorders [1]. In a normal situation, scar formation is an important mechanism to repair tissue damage. Upon chronic injury, however, scar tissue deposition is excessive up to a point where normal liver functions are compromised [2][3]. Over time, this process can lead to cirrhosis, a phase in which the architecture and function of the organ is so disrupted that serious complications can occur and ultimately this may result in complete liver failure and death [4]. Regardless of the cause of injury, damaged hepatocytes, activated Kupffer cells and liver endothelial cells release fibrogenic stimuli that promote the activation of HSC [2,5]. Deposition of matrix components produced by HSC, the key cell type in promotion of liver fibrosis, builds up non-functional connective tissue in the liver, accompanied by reduced collagenolysis [3,6]. Removing the primary cause of the disease might slow down fibrosis progression, but does not provide a cure or a complete reversion of fibrosis [7][8,9]. Currently, there are no antifibrotic drugs available and the only option to prolong life in patients with advanced fibrosis is a liver transplantation. The recognition that activated HSC are the major fibrogenic cell type in the liver [10][11-13] and the identification of several cytokines and pathways that are involved in the progression of hepatic fibrosis, may lead to promising new antifibrotic therapies [14][15-19]. However, these approaches have not reached the clinic yet mostly because of low efficacy and safety in humans [20].

Interleukin-10 (IL-10) is known for being a potent anti-inflammatory cytokine [21,22]. Based on several studies that demonstrated antifibrotic effects of endogenous IL-10 [23][24,25], a clinical trial with IL-10 in patients with hepatitis C-induced liver fibrosis was conducted. These studies yielded promising results, but a flare-up of the virus, associated with an immunosuppressive effect due to prolonged treatment with IL-10, led to the withdrawal of the trial [26][27]. In recent years, many inconsistent and controversial studies on the role of IL-10 during fibrosis have been published, showing either pro-or anti-fibrotic effects of IL-10 depending on the organ and/or the animal model used [28-30]. One reason for these disappointing results may be short plasma half-life of IL-10 due to the rapid renal clearance of this low molecular

weight protein [31]. Long term administration of IL-10 may not be the best option because of the multiple off-target effects in various tissues, as observed with many other cytokines [32][33][26][34][26,35]. Another hypothesis for the conflicting results of systemically administered IL-10 may be that different cells in a specific organ respond differently to IL-10. The final outcome of the IL-10 treatment would then be the mixed response of different cells, leading to variable results in different conditions. It is known for instance, that IL-10 has anti-fibrotic effects in HSC [36][37], but pro-fibrotic effects in some macrophage subtypes [38][39], and it attenuates the inflammatory process that is commonly associated with fibrosis, thus affecting liver fibrosis in many ways. These pleiotropic effects are extremely difficult to untie *in vivo* but insight in this is essential to understand the outcome of different experimental studies and clinical trials. To examine the direct role of IL-10 during fibrogenesis we therefore applied a cell-specific delivery approach for IL-10, thus mimicking local production of this cytokine.

In the present thesis, we describe the successful preparation of a conjugate that is selectively delivered to HSC *in vivo* during liver fibrogenesis in mice. IL-10 was chemically modified with a cycle peptide (PPB) that binds to the (overexpressed) PDGFβ receptor on activated HSC. A PEG linker of 2 kDa between IL-10 and this peptide was used to provide stability and flexibility to the molecule. Our studies on the effects of this conjugate *in vivo* yield new insights into the direct and indirect effects of IL-10 on HSC and provide evidence for cross-talk between these cells and liver macrophages. The targeting of IL-10 or other biological mediators to different cells within the liver may present new opportunities for the development of novel therapies to treat liver fibrosis in the future.

As outlined in **chapter 1** (general introduction), IL-10 plays an important role in both progression and resolution of fibrosis in several organs. As hepatic fibrosis is a very complex disorder, involving the intricate participation of extracellular matrix components, hepatic cells and a variety of endogenous substances, the elucidation of these interactions and pathways could be translated into new drugs for anti-fibrotic therapies. Our study provides such a step.

The short half-life of IL-10, due to rapid renal clearance, is a major limitation that hampers the use of this cytokine for the treatment of a chronic disease like liver fibrosis [40]. PEGylation is a well-known technique that is widely used to prolong the half-life of peptides and proteins in plasma and thus to enhance their therapeutical potency [41-44]. We therefore modified IL-10 with polyethylene glycol (PEG) with the purpose of to prolong its circulation time and to enhance its effects. **Chapter 3** shows the chemical modification of IL-10 with either 5 or 20 kDa PEG. The two compounds were compared with each other and with native IL-10 in mice with CCl_4-induced liver fibrosis focusing on their circulation time, liver accumulation and effect

on several fibrotic parameters. PEGylation might affect the binding capacity of the cytokine to its receptor and therefore the biological effects of this protein might be compromised [45]. However, our results showed that the biological activity of IL-10 after PEGylation was preserved. Subsequently we measured the pharmacokinetic profile of the newly synthesized conjugates and our data revealed a longer circulation time and higher liver accumulation of both forms PEGylated IL-10 compared to unmodified cytokine. To our knowledge, our study is the first to describe the plasma profile and effectivity of a PEGylated form of IL-10 after systemic administration. A PEGylated form of IL-10 has been previously produced and tested against neuropathic pain, but it was only used locally [44]. *In vivo* effect studies of both PEGylated forms of IL-10 in an acute CCl_4-induced liver damage model in mice revealed that IL-10 PEGylated with 5kDa PEG (5PEG-IL10) was more effective in reducing liver inflammation and fibrosis than 20PEG-IL10 or unmodified IL-10. However, it was not clear whether the decrease in inflammation and fibrosis was a direct effect of IL-10 on HSC or an indirect effect via inhibition of Kupffer cell activation, because both HSC activation (as shown by downregulation of α-SMA) and macrophage activation were affected by 5PEG-IL10. The altered macrophage activation was reflected by a decreased MHC class II expression and an increased expression of the macrophage marker FOXP3, in 5PEG-IL10 treated animals. This particular change in profile of macrophages suggested that 5PEG-IL10 induced a polarization of macrophages into the M2c subtype, also known as regulatory macrophages, which represent a de-activated subtype of macrophages. Alternatively, an effect via systemic down regulation of the immune response by this immunosuppressive cytokine cannot be excluded. In view of the direct anti-fibrotic effects of IL-10 found in cultures of HSC [15,46,47], we set out to deliver IL-10 to this particular cell type.

In order to evaluate the best option for HSC-specific delivery we first explored two different carriers to activated HSC, that have been developed in the past years within our group. We tested delivery of compounds to HSC via 1) the Mannose-6-phosphate/Insulin-like Growth Factor II (IGFII) receptor using human serum albumin (HSA) modified with mannose-6-phosphate (M6PHSA) and via 2) the PDGFβ-receptor using HSA substituted with PDGFβ-Receptor recognizing peptides (PPBHSA). Both IGFII receptors and PDGFβ-receptors are highly and selectively upregulated on activated HSC and the selectivity of both carriers has been extensively demonstrated in previous studies. Both carriers have been previously used to deliver therapeutic compounds to this cell type in the fibrotic liver [48-52]. We used the carriers to deliver 15-deoxy-δ12,14-prostaglandin J2 ($15dPGJ_2$) to HSC, a compound known to induce apoptosis in this cell type [53]. The coupling of $15dPGJ_2$ to the drug carriers M6PHSA and PPBHSA led in both cases to a rapid and high accumulation of the

prostaglandin uptake in the fibrotic liver. Up to 80% was found back in the liver, which represents massive uptake in both cases. Both targeted forms of 15dPGJ$_2$ kept their biologic activities *in vitro* and both types of constructs accumulated within HSC of fibrotic livers. However, the cellular binding of 15dPGJ2-M6PHSA was reduced by a scavenger receptor antagonist and intrahepatic investigation revealed that this conjugate was also found in Kupffer cells, known to express a scavenger receptor [54,55] [56][57]. Targeting to the PDGFβ- receptor on HSC appeared to be a better option because selective uptake in HSC was found, with no uptake in other non-parenchymal cells of the liver and in our studies it was crucial to prevent uptake of IL-10 in KC. Therefore we decided to use PPB to modify IL-10 and to deliver this cytokine to the HSC in the fibrotic liver. We hypothesized that IL-10 receptor triggering while targeting to the PDGFβ-receptor can be achieved by receptor cross-talk. Binding of the conjugate to the PDGFβ-receptor, which is abundantly expressed on activated HSC relative to IL-10-receptor expression, will lead to high accumulation of IL-10 on the plasma membrane of HSC. The flexible (PEG) linker between the PDGF-binding peptide and the IL-10 molecule may allow occasional binding of IL-10 to its receptor either in a nearby location of the same cell or on a neighboring HSC, thereby inducing IL-10-R-mediated effects.

Some groups have described IL-10 as a cytokine endowed with direct antifibrotic effects *in vitro* in HSC by preventing the activation of this cell type [23,58-60], and by downregulation of TIMP-1 and consequently promoting the degradation of collagen types I and III. However, *in vivo* data have shown disappointing or even conflicting results, and IL-10 administration even elicited significant pro- fibrotic responses in some organs (Lee, 2002; Sun, 2011). To unravel the specific effects of IL-10 on HSC during hepatic fibrogenesis we therefore chemically modified IL-10 with PPB, using 2kDa PEG as a linker (IL10-PEG-PPB), as described in **chapter 4.** Results of chapter 3 had indicated to us that even a short PEG molecule significantly enhanced the circulation time of IL-10 in plasma, without affecting its receptor interaction. PPB-was conjugated to enhance delivery to the PDGF-β receptor. Delivery of albumin to the PDGFβ –receptor using this PPB peptide has been demonstrated extensively in previous studies [61][62,63]. The conjugate was characterized and tested *in vitro* for its biologic activity, confirming its receptor interaction. *In vivo* effects were then explored in mice with CCl_4-induced fibrogenesis. Much to our surprise, an increased deposition of Collagen type I was found in IL10-PEG-PPB-treated animals. This was associated with a significant upregulation of TIMP-1 compared to untreated or IL-10-treated mice and a reduced synthesis of the matrix degrading metalloproteinase MMP-13, all pointing towards reduced collagen degradation. It is known that TIMP and MMP-13 are significantly produced by macrophages which prompted us to explore these cells further. We found a significant increased influx of the M2 subset of

macrophages in livers of IL10-PEG-PPB-treated animals, as reflected by the markers YM1 and FOXP3. So, whereas systemic delivery of IL-10 attenuated liver fibrosis in this inflammatory model in mice and prolongation of its circulation time enhanced this antifibrotic effect (chapter 3), local delivery of IL-10 to HSC led to enhanced fibrosis associated with an enhanced influx of M2-macrophages with pro-fibrotic activities. So, despite delivery of IL-10 to HSC, we found major effects on macrophage activities. To elucidate the role of IL-10 in the cellular communication between HCS and liver macrophages during fibrogenesis *in vivo* and to unravel the mechanism by which selective targeting of IL-10 to HSC triggers fibrogenesis, we pursued our research in the same mice model (**chapter 5**). In this study, other parameters, such as collagen type III, fibronectin and TGFβ, confirmed the profibrotic effect of specific targeting of IL-10 to HSC. Furthermore, we found upregulation of CCR2 receptor expression in these livers, associated with an upregulation of its ligand CCL2. This CCR2/CCL2-axis is known to have chemotactic activities for macrophages and fibroblast-like cells [64][65][66], and a recent study showed upregulation of this chemotactic factor in HSC during lung fibrogenesis [67]. Our study therefore indicates that IL-10, delivered to HSC, is involved in the recruitment of macrophages to the fibrotic liver via upregulation of the CCR2/CCL2 axis on HSC. This subsequently leads to accumulation of regulatory macrophages (M2c subtype) in these livers. Our study provides therefore evidence for a cell-cell communication between HSC and KC or macrophages involving IL-10 and the CCL2/CCR2 axis. This opens possibilities to modify macrophage influx and macrophage polarization in fibrotic livers thereby modifying this pathological process.

Conclusions and future perspectives

In summary, the **aim** of the studies described in this thesis focuses on the role of IL-10 in hepatic fibrosis, especially on its specific effects on hepatic stellate cells (HSC) *in vivo* by means of a cell-specific targeting technique. Taken together, our results suggest that IL-10 triggers different effects depending on the cell type that it is binding to.

Upon PEGylation, the circulation time was prolonged, which was associated with an increased accumulation in the liver and hence an enhanced anti-fibrotic effect during CCl_4-induced liver fibrosis. The therapeutical application of IL-10 during liver fibrosis was hampered in clinical trials by the pleiotropic effects of IL-10 in other cells, tissues or organs. In particular the systemic immunosuppressive effects prevented chronic administration to patients [26], and even caused cessation of the trials, and obviously these effects are not prevented after prolongation of its

circulation time after PEGylation. Since macrophages are known to express more IL-10 receptors than HSC [68], it seems logical that PEGylated IL-10 affects liver macrophages in a more pronounced way than HSC. Specific targeting of IL-10 to the PDGFβ receptor may therefore be a good option to target IL-10 to the activated HSC. Upon activation, HSC are characterized by a high expression of the PDGFβ receptor thus facilitation binding and uptake of our conjugate in these cells within the fibrotic liver. The low amounts of IL-10 administered to mice prevented to study binding and uptake *in vivo* in detail, but this drug-targeting concept has been extensively demonstrated with albumin as a core molecule. Surprisingly, while systemic IL-10 induced anti-fibrotic effects (chapter 3), HSC-targeted IL-10 triggered a profibrotic response in fibrotic livers (chapter 4 and 5). Macrophages and KC do not express the PDGF-receptor and uptake of PPB-constructs in KC has never been observed. Apparently, bypassing the macrophage by specific delivery to HSC completely changes the activity of IL-10. Nevertheless, significant changes in macrophage polarization were noted after delivery of IL-10 to HSC, suggesting an indirect effect of this IL-10 on the macrophage population via modification of HSC responses. In recent reports, the CCL2/CCR2 axis was hypothesized as being responsible for the recruitment of fibrogenic cells into the lungs and [69], and we found that the macrophage phenotypic profile in the liver shifted towards an M2c subtype, which is known to be endowed with pro-fibrotic activities [70][71]. Collectively, our data indicate that IL-10 locally produced during fibrogenesis affects HSC thereby creating a pro-fibrogenic response, possibly via the CCL2/CCR2 axis and macrophage polarization into the M2-subtype, whereas it also affects the macrophage population thereby creating an anti-fibrogenic response possibly via modification of the MMP-13/TIMP-1 balance. The composition of cells during the time of IL-10 administration and the route of administration (locals versus systemic) determines the net effect of IL-10. This would also explain the variable results seen in many studies on the effects of IL-10 during fibrosis in different organs and different experimental models.

This is the first study demonstrating that specific targeting of IL-10 to HSC *in vivo* leads to effects on the development of hepatic fibrosis associated with macrophage polarization. Hepatic fibrosis is a very complex pathological process and up to now there is no treatment available for any kind of fibrosis, so the development of cell-specific, re-directed cytokines or other biological drugs may provide a suitable approach to both untie the intricate pathophysiology of this disease and serve as a basis for novel anti-fibrotic therapies. In future studies, the targeting of IL-10 to other hepatic cell types, in particular to liver macrophages, may provide a new world of knowledge about the pathogenesis of hepatic fibrosis and it might prove to be useful for the development of effective therapies for this chronic disease.

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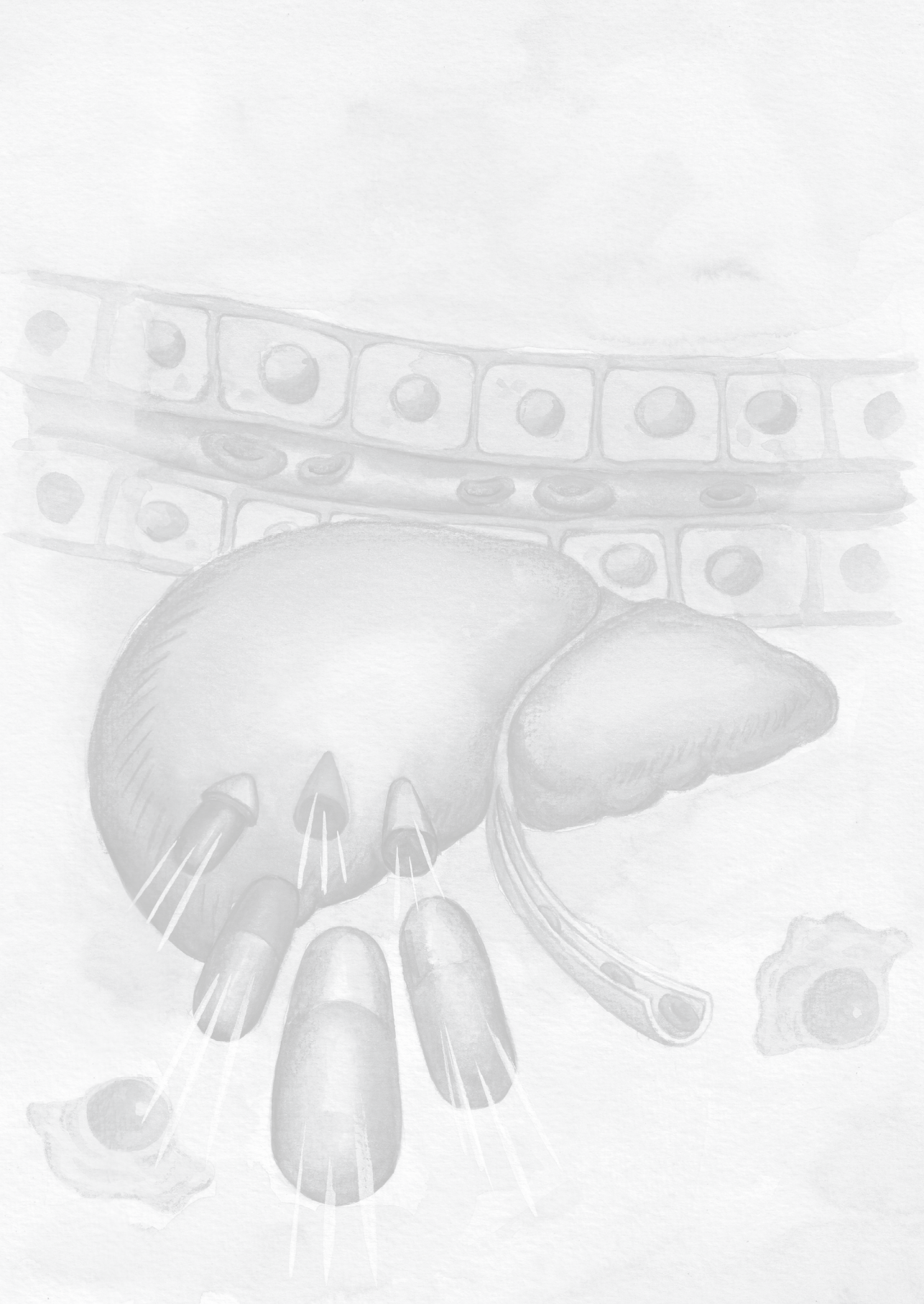


Chapter 7

Samenvatting and resumo

Samenvatting

Dit proefschrift beschrijft de effecten van een cel-specifieke aflevering van Interleukine-10 in de Hepatische Stellaat cel. Cytokines zoals Interleukine-10 zijn boodschapper eiwitten die een cruciale rol spelen in de communicatie tussen cellen onderling. Interleukine-10 regelt een aantal belangrijke maar zeer uiteenlopende functies tijdens ontstekingen: het remt de ontstekingsactiviteit door een remmend effect op macrofagen en andere ontstekingscellen en het remt tegelijkertijd littekenvorming door een direct effect op fibroblast-achtige cellen. Hepatische stellaat cellen zijn fibroblast-achtige cellen in de lever die een cruciale rol spelen bij weefselherstel tijden leverfibrose. Lever fibrose komt wereldwijd voor in miljoenen patiënten. Na leverschade door hepatitis virussen, alcohol misbruik, het metabool syndroom (gerelateerd aan obesitas), auto-immuunziektes of een aantal genetische ziektes ontstaat schade aan het lever parenchym. Deze schade wordt direct hersteld waarbij littekenweefsel (o.a. collageen) wordt afgezet in de beschadigde gebieden. Dit littekenweefsel wordt vooral geproduceerd door Hepatische stellaat cellen. In een normale lever zijn deze cellen in rust (en slaan vooral Vitamine A op), maar na activatie door een ontstekingsproces of lokale beschadiging verliezen deze cellen hun vitamine A en gaan ze collagenen en andere extracellulaire matrix eiwitten aanmaken. Het worden dan fibroblast-achtige cellen. Dit is uiteraard zeer nuttig maar chronische stimulatie van dit proces kan leiden tot een verstoorde regulatie van extracellulaire matrix vorming en afbraak waardoor er een zichzelf stimulerend proces op gang komt, uiteindelijk leidend tot een overmatige afzetting van littekenweefsel in de gehele lever. Uiteindelijk zal dit leiden tot leverfalen in het eindstadium (lever cirrhose). Hiervoor is nog geen therapie beschikbaar, alleen een levertransplantatie is mogelijk. Het moge duidelijk zijn dat dit geen oplossing is voor iedereen en nieuwe geneesmiddelen om dit proces te remmen zijn dan ook dringend gewenst. In onze studies hebben wij getracht gebruik te maken van lichaamseigen stoffen die betrokken zijn bij de regulatie van het ziekteproces. Het proces van leverfibrose kan wel 10 tot 30 jaar duren, hetgeen al aangeeft dat er ook krachtige remmers actief zijn tijdens dit ziekteproces. Wij besloten gebruik te maken van deze lichaamseigen remmers en kwamen daarbij uit op Interleukine-10.

In het verleden is Interleukin-10 al eens getest in een klinische trial in patiënten met leverfibrose. Een groot deel van deze patiënten had echter Hepatitis B of C als onderliggende oorzaak van leverfibrose en het chronische gebruik van Interleukine-10 veroorzaakte een (re-)activatie van de virale ontstekingsactiviteit. Deze klinische trial werd daarom beëindigd. De reactivatie van de virale infectie werd hoogstwaarschijnlijk veroorzaakt door het ontstekingsremmend effect van Interleukine-10. Door remming van Macrofagen en T en B cellen ontstaat een sterke immunosuppressie

waardoor het virus weer een kans krijgt. Wij besloten daarop om het Interleukin1-10 zodanig te veranderen dat het alleen in de lever actief is en het immuunsysteem niet aangetast wordt.

De bestaande literatuur liet zien dat het effect van interleukine-10 tijdens het fibrose proces zeer complex is. In **Hoofdstuk 1** hebben we daarvan een overzicht gegeven. Sommige studies lieten een remmend effect zien, andere een stimulerend effect op het ziekte proces. Interleukine 10 heeft verschillende, soms zelfs tegenstrijdige effecten op verschillende cellen in het zieke weefsel. Wij besloten daarom twee strategieën te testen: (1) door het eiwit te Pegyleren (= te voorzien van poly-ethyleen glycol) waardoor overlevingstijd van eiwitten in het bloed sterk verlengd wordt en daarmee de kans verhoogd wordt dat Interleukine-10 het zieke weefsel bereikt en (2) door het cytokine gericht te sturen naar de cellen die het belangrijkste zijn bij het ontstaan van leverfibrose: de hepatische stellaat cellen.

De overlevingstijd van cytokines in het bloed is meestal vrij kort (zo ook van Interleukine-10) waardoor het cytokine het doel orgaan in onvoldoende mate bereikt. Cytokines worden normaliter lokaal geproduceerd en hun belangrijkste effecten zijn ook vaak lokaal. Opruimmechanismen in het lichaam zorgen ervoor dat cytokines ook vaak alleen lokaal actief zijn en juist dit blokkeert het systemisch therapeutisch gebruik van de meeste cytokines. Pegylering van diverse cytokines (met name Interferonen) bleek al zeer succesvol: door de verlengde overlevingstijd kwam er bijvoorbeeld meer Interferon α2 (a en b) in de lever terecht en werden de antivirale effecten van deze cytokines in de lever versterkt. Op het gebied van de bestrijding van Hepatitis heeft dit tot een doorbraak geleid. Wij hebben daarom eerst bij Interleukine-10 dezelfde strategie toegepast. Deze studies zijn beschreven in **Hoofdstuk 3**. Pegylering van Interleukine 10 verlengde sterk de halfwaardetijd in bloedplasma en leidde tot een significant verhoogde accumulatie in de lever en een significante verbetering van de anti-fibrotische activiteit in de fibrotische lever bij muizen ten opzichte van onveranderd Interleukine-10. We hebben in onze studies twee verschillende molecuul groottes getest (PEG 5 Kd en PEG 20 Kd). Uit onze resultaten bleek dat het kleinste PEG molecuul al grote effecten had op de overlevingstijd in plasma en niet de binding van Interleukine-10 aan zijn receptor verstoorde. De anti-fibrotische effecten van PEG_{5kd}-Interleukine-10 bleken in het proefdiermodel significant beter ten opzichte van onveranderd Interleukine-10 en PEG_{20kd}-Interleukine-10. We concludeerden dat PEGylering van Interleukine-10 een zinvolle strategie is en leidt tot anti-fibrotische effecten in ons muis model.

Daarnaast hebben we het effect van een cel-specifieke aflevering van Interleukine-10 onderzocht. De doelcel was de geactiveerde hepatische stellaat cel, omdat deze cel de belangrijkste bron van extracellulaire eiwitten is in de fibrotische lever en deze cel ook de activiteit van andere cellen (andere fibroblasten, macrofagen

e.d.) kan stimuleren en daardoor het fibrose proces op gang kan houden. Er zijn directe effecten van Interleukine-10 op de hepatische stellaat cel beschreven, resulterend in een remming van de collageen producerende activiteit. De hepatische stellaat cel brengt na activatie een aantal receptoren in verhoogde mate tot expressie die kunnen dienen als doelreceptor om geneesmiddelen af te leveren. De Insulin-like Growth Factor II receptor (IGFII-R) en de Platelet Derived Growth Factor Receptor (PDGF-R)zijn de meest onderzochte voorbeelden hiervan. Wij hebben liganden ontwikkeld die binden aan een van deze twee receptoren en daarmee hebben we geneesmiddeldragers ontwikkeld die specifiek geneesmiddelen in de hepatische stellaat cel kunnen afleveren. Deze geneesmiddeldragers bestaan uit een groot eiwit (in ons geval albumine) met daaraan gekoppeld mannose-6-phosphaat (M6P) dat bindt aan de IGF-II receptor of een peptide (pPB) dat bindt aan de PDGF-receptor. Beide accumuleren in hoge mate in de geactiveerde hepatische stellaat cellen tijdens leverfibrose. Ons doel was om Interleukine-10 te koppen aan een van deze receptor bindende liganden en daarmee af te leveren in de hepatische stellaat cel. Wij hebben allereerst onderzocht welke doel receptor het meest geschikt was voor onze doeleinden. Deze studies zijn beschreven in **hoofdstuk 2**. In deze eerste studies hebben we een ander endogene remmer van fibrose aan de geneesmiddel drager gekoppeld: 15-d-deoxy-Prostaglandine J_2. Uit de literatuur bleek dat dit prostaglandine apoptosis (geprogrammeerde celdood) kan induceren in hepatische stellaat cellen en daarmee ook een anti-fibrotisch effect kan uitoefenen. Prostaglandines hebben in het algemeen ook een zeer korte halfwaarde tijd in bloed, reden voor ons om te testen of deze stof ook specifiek is af te leveren in deze doelcellen. Deze studies toonden aan dat beide geneesmiddel constructen selectief ophoopten in de hepatische stellaat cel. Maar liefst 80% van de geïnjecteerde dosis kwam in de fibrotische lever terecht en daarvan het merendeel in de hepatische stellaat cel. Een deel van de constructen kwam echter in de Kupffer cellen en endotheel cellen terecht waarbij deze percentages bij de M6P-constructen iets hoger lagen dan van de pPB-constructen. Voor onze studies was het belangrijk om de Kupffer cel te ontwijken omdat dit zou kunnen leiden tot immunosuppressieve effecten die juist vermeden moesten worden bij Interleukine-10 therapieën. We besloten daarop om het PDGF-R bindende peptide pPB te gebruiken voor de cel-specifieke aflevering van Interleukine-10 in Hepatische stellaat cellen. Op grond van de resultaten beschreven in Hoofdstuk 3 besloten we daarnaast ook om een klein PEG-molecule te gebruiken (2 kD) voor de koppeling van pPB aan het Interleukine-10.

De effecten van pPB-PEG-Interleukine-10 zijn vervolgens bestudeerd in een muis model. Deze studies zijn beschreven in **hoofdstuk 4 en 5** van dit proefschrift. We hebben een chronisch model voor lever fibrose gebruikt waarbijgedurende 8 weken CCl4 wordt toegediend. De laatste 2 weken ontvingen de muizen een behandeling

met Interleukine-10 of modificaties daarvan. De constructen werden eerst in vitro op fibroblast-achtige cellen getest en bleken goed te werken. De synthese procedure en het afleveren op de doelreceptor had de biologische activiteit dus niet aangetast. In vivo, in het proefdiermodel, bleek dit gericht gestuurde interleukine-10 echter geen anti-fibrotische effecten te bezitten maar sterk profibrotische effecten (**Hoofdstuk 4**). Dit was niet verwacht. Parallel zagen we daarbij duidelijke veranderingen in macrofagen: er bleken meer macrofagen aanwezig te zijn in levers van proefdieren die waren behandelend met PPB-PEG-Interleukine-10 dan in levers van zieke dieren die niet waren behandeld met enig eiwit of die onveranderd Interleukine-10 hadden gekregen. Deze macrofagen bleken van een bepaald subtype (M2c) waarvan recent was aangetoond dat deze het wondgenezingsproces versnelde door de collageen opbouw te stimuleren en de afbraak te verminderen. Dit zou de toename van collageen afzetting in onze studies kunnen verklaren. Omdat we het Interleukine-10 naar de hepatische stellaat cel hadden gestuurd maar veranderingen zagen in macrofagen (terwijl deze geen PDGF-receptor bezitten) hebben we onze vervolgstudies (beschreven in **hoofdstuk 5**) gericht op de interactie tussen hepatische stellaat cellen en macrofagen. Uit deze studies bleek dat in de levers van muizen die behandeld waren met PPB-PEG-Interleukine-10 een verhoogde aanwezigheid was van stoffen die macrofagen aantrekken (CCL2) evenals de receptoren voor deze chemotactische factor (CCR2). In de literatuur werden studies gepubliceerd die aantoonden dat hepatische stellaat cellen macrofagen kunnen aantrekken door productie van CCL2. Onze data suggereren dat dit leidt tot een ophoping van macrofagen van het M2c subtype. Dit subtype produceert onder andere Transforming Growth Factor β, een pro-fibrotische groeifactor. Op grond van de resultaten van Hoofdstuk 4 en 5 luidt onze hypothese dan ook dat Interleukinie-10 een sturende rol speelt in fibroblasten: dit cytokine stimuleert de productie van CCL2 door hepatische stellaat cellen waardoor macrofagen (positief voor CCR2) worden aangetrokken. Deze macrofagen stimuleren op hun beurt het wondgenezingsproces met als gevolg een verhoogde collageen afzetting. Dus terwijl onveranderd Interleukine-10 een anti-fibrotisch effect heeft dat versterkt wordt door een verlenging van de halfwaardetijd in plasma, is het netto-effect van Interleukine 10 in hepatische stellaat cellen een profibrotisch effect.

Wij concluderen in **hoofdstuk 6** dat door onze studies veel duidelijk is geworden over de rol van Interleukine 10 tijdens leverfibrose. Interleukine-10 beïnvloedt het fibrose proces op verschillende manieren: intraveneuze toediening leidt tot anti-fibrotische effecten mogelijk via een ontstekingsremmend effect of andere celtypen, terwijl aflevering bij de hepatische stellaat cel leidt tot pro-fibrotische effecten via het aantrekken van macrofagen. De literatuur over interleukine-10 is tegenstrijdig en onze bevindingen zouden veel daarvan kunnen verklaren. Het effect van Interleukine -10 is afhankelijk van het celtype dat domineert in de zieke lever;

ontstekingscellen, macrofagen dan wel hepatische stellaat cellen kunnen de overhand hebben. Deze samenstelling kan aanzienlijk verschillen tussen een vroege fase (met veel ontstekingsactiviteit) en een late fase van de ziekte (met veel geactiveerde hepatische stellaat cellen). Ook in de diverse diermodellen die gebruikt worden in de literatuur verschilt de aanwezigheid van ontstekingscellen, macrofagen en hepatische stellaat cellen sterk. Onze studies suggereren ook een belangrijke sturende rol voor M2c macrofagen tijdens het fibrose proces. Mogelijk is het gericht sturen van stoffen naar de M2c macrofagen in de lever, om deze cellen te remmen in hun activiteit een geschikte anti-fibrotische therapie maar dit moet verder onderzocht worden. Onze studies laten daarnaast zien dat het gericht sturen van cytokines naar bepaalde doelcellen zeer goed mogelijk is en ook dit vormt een basis voor nieuwe kansen voor de bestrijding van deze veelvoorkomende chronische ziekte.

Resumo

Esta tese descreve os efeitos de um endereçamento específico de Interleucina-10 (IL-10) para células estreladas hepáticas (HSCs) durante o processo fibrótico. As citocinas, como por exemplo a Interleucina-10, são proteínas mensageiras que desempenham um papel fundamental na comunicação intercelular. A Interleucina-10 apresenta uma variedade de funções, sendo muito importante durante a inflamação porque tem ação inibitória sobre os macrófagos e outras células envolvidas no processo inflamatório. Outra propriedade bastante conhecida da IL-10 é a de diminuir a formação de tecido cicatricial por ter um efeito inibitório direto sobre fibroblastos. As HSCs são fibroblastos que se localizam no fígado e desempenham um papel crucial na reparação de tecidos durante o processo fibrótico. A fibrose hepática é uma doença que afeta milhões de pessoas ao redor do mundo e pode ocorrer após lesão crônica que causa danos ao parênquima do fígado, podendo ser causada por vírus da hepatite, abuso de álcool, síndrome metabólica (relacionada à obesidade), doenças auto-imunes e algumas doenças genéticas. Este dano é imediatamente reparado com tecido cicatricial (colágenos, por exemplo) que é depositado nas áreas danificadas. O tecido cicatricial é produzido principalmente por HSCs que, em um fígado normal, estão em estado quiescente e armazenam alguns tipos de lipídios, principalmente vitamina A. Quando inicia-se um processo inflamatório ou injúria tecidual, essas células passam a um estado ativado, assumindo um fenótipo semelhante ao de um fibroblasto, perdem o estoque de vitamina A e passam a produzir colágenos e outras proteínas da matriz extracelular em excesso. O mecanismo fisiológico de reparo tecidual é muito importante na manutenção da homeostase, mas seu excesso conduz a um desequilíbrio entre a formação da matriz extracelular e sua degradação. Como consequência dá-se a formação excessiva de tecido cicatricial por todo o fígado, o que acaba por comprometer o funcionamento normal do órgão, levando à insuficiência hepática conhecida por cirrose. Não existe terapia eficaz disponível para a cirrose hepática e a única forma de tratamento é o transplante de fígado. Assim sendo, novos medicamentos capazes de tratar essa patologia são extremamente necessários. No presente estudo fizemos uso de substâncias naturalmente produzidas pelo organismo e que estão envolvidas na regulação do processo fibrólise-fibrogênese. A fibrose hepática é uma doença de progressão lenta, podendo levar de 10 a 30 anos para se desenvolver após o início da injúria crônica, o que indica que existem inibidores ativos durante o desenvolvimento dessa patologia. Um desses inibidores endógenos é a Interleucina-10, que foi usada durante os estudos na presente tese.

Os efeitos da administração da Interleucina-10 foram avaliados em uma triagem clínica realizada em pacientes com fibrose hepática causada por hepatites dos tipos B e C. O uso prolongado de IL-10 causou, em grande parte dos pacientes, a reativação

da infecção viral e a triagem foi, portanto, encerrada. Provavelmente a causa da reativação da atividade viral foi devida ao efeito anti-inflamatório de IL-10 e a inibição de linfócitos B e T que criaram um ambiente de imunossupressão, favorecendo o ressurgimento da fase infecciosa. Por esse motivo, decidimos re-direcionar a IL-10 para o fígado e, desse modo, tentar não comprometer o sistema imunológico do organismo.

Dados da literatura mostram que o papel da IL-10 durante o processo fibrótico é muito complexo e o **capítulo 1** desta tese nos dá uma visão geral sobre esse assunto. Os efeitos da IL-10 podem muitas vezes ser distintos em diferentes células de um mesmo tecido afetado pela doença, ora agindo como inibidor, ora como estimulante. Tendo isso em vista, decidimos testar duas estratégias: (1) aumentar o tempo de circulação da proteína no sangue, por meio de acoplamento com polietileno glicol (PEG), aumentando assim a chance da IL-10 de chegar ao tecido afetado e (2) endereçar a IL-10 especificamente para as HSCs que são as células-chave na patogênese da fibrose hepática.

O tempo de circulação de citocinas no sangue, assim como o da IL-10, é geralmente bastante curto e, desse modo, o órgão-alvo é exposto de maneira insuficiente. As citocinas geralmente agem no local onde são produzidas e secretadas o que, por si só, já é suficiente para limitar seu uso terapêutico sistêmico. A técnica de peguilação das citocinas, em particular dos interferons, é muito eficaz, pois prolonga sua meia-vida no plama. Como exemplo podemos citar os Interferons-α2 (A e B) cujos efeitos anti-virais no fígado foram potencializados por causa da peguilação, o que propiciou um enorme avanço na luta contra as hepatites virais. Pela primeira vez utilizamos essa estratégia com a Interleucina-10 e os resultados desses estudos estão descritos no **capítulo 3** desta tese. A peguilação da IL-10 prolongou significativamente sua meia-vida no plasma, favorecendo seu expressivo acúmulo no fígado e levando a uma melhoria da fibrose hepática em camundongos. Dois tamanhos diferentes de moléculas de PEG foram testados nesse artigo (5kDa e 20kDa). Os resultados mostraram que a molécula de PEG de menor peso foi a que obteve os melhores resultados tanto em relação ao aumento do tempo de circulação sanguínea quanto à manutenção da atividade biológica da citocina. Os efeitos anti-inflamatórios e anti-fibróticos do composto 5PEG-IL-10 no modelo animal foram significativamente melhores em relação à IL-10 não modificada e ao composto 20PEG-IL-10. Concluímos então que a peguilação de IL-10 é uma estratégia útil que conduz a efeitos anti-fibróticos em nosso modelo murino.

Depois desse estudo, decidimos examinar o efeito da IL-10 em um tipo específico de célula. A célula-alvo escolhida foi a forma ativada das células estreladas hepáticas (HSCs), uma vez que esse tipo celular representa a principal fonte de proteínas extracelulares no fígado fibrótico, além de estimular a atividade pró-fibótica em outras células (outros fibroblastos, macrófagos etc), e, por conseguinte, é responsável por manter o processo de fibrose ativo.

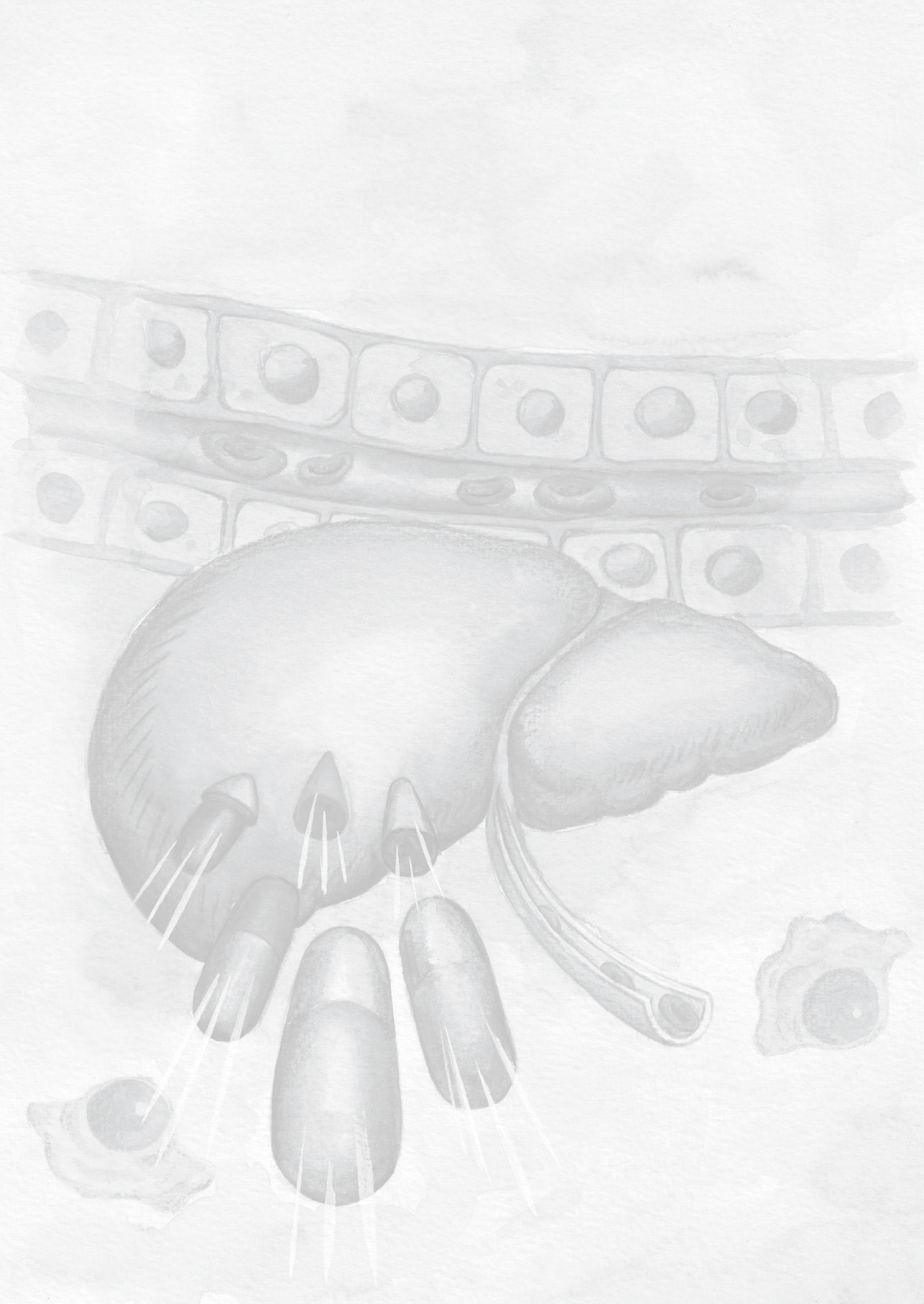
Existem artigos descrevendo efeitos diretos da IL-10 nas células estreladas hepáticas, com diminuição da produção de colágenos. Quando as HSCs são ativadas, passam a expressar um excesso de certos receptores que podem servir como receptores-alvo para o endereçamento de drogas. Como exemplos mais estudados podemos citar o receptor do fator de crescimento similar à insulina tipo 2 (IGFII-R) e o receptor do fator de crescimento derivado de plaquetas (PDGF-R). Em nosso laboratório foram desenvolvidos ligantes que têm afinidade por esses receptores e que funcionam como veículo de endereçamento específico de fármacos para as HSCs, que apresentam esses receptores em excesso quando ativadas. Os conjugados transportadores de fármacos são constituídos por uma proteína de grande peso molecular (nesse caso, a albumina) que tem acoplada à sua estrutura manose-6-fosfato (M6P), que vai se ligar ao receptor IGF-II, ou um peptídeo (PPB) que irá se unir ao receptor PDGF. Ambos os conjugados acumulam-se em grande quantidade em HSCs ativadas durante a fibrose hepática. Em nosso estudo o objetivo foi o de conjugar a IL-10 a um desses ligantes específicos e endereçar o conjugado às células estreladas hepáticas. No **capítulo 2** são descritos experimentos e resultados que nos possibilitaram determinar o receptor-alvo mais apropriado para atingir nossos objetivos. Inicialmente, utilizamos outra droga biológica na síntese dos conjugados de endereçamento: a prostaglandina 15-d-PGJ_2 (15-desoxi-D12,14-Prostaglandina J2), já que dados de literatura indicam que esta é indutora de apoptose (morte celular programada) em HSCs e que produz, portanto, um efeito anti-fibrótico. Em geral, as prostaglandinas têm meia-vida plasmática muito curta, o que já é motivo suficiente para a realização de testes no sentido de se determinar os efeitos destas quando fornecidas diretamente às células-alvo. Os resultados desse artigo mostraram que os conjugados com a droga biológica acumularam-se seletivamente em HSCs, com cerca de 80% da dose inicial injetada com localização em áreas fibróticas do fígado e, em sua maioria, co-localizadas com HSCs. No entanto, o conjugado M6P se acumulou mais do que o conjugado PPB em células endoteliais e de Kupffer. Para nossos estudos com IL-10 é importante evitar o acúmulo em células de Kupffer, já que isso poderia induzir efeitos imunossupressores relatados em terapias com Interleucina-10. Com base nesses resultados resolvemos que o endereçamento da IL-10 para o receptor PDGF seria a estratégia mais adequada para o envio de IL-10 às HSCs ativadas durante o processo fibrótico. Tendo como base os resultados descritos no capítulo 3, decidimos utilizar uma molécula PEG de baixo peso (2kDa) como ligante entre o PPB e a IL-10 no conjugado.

Os efeitos do novo conjugado (PPB-PEG-IL-10) em camundongos, foram descritos nos **capítulos 4 e 5** desta tese. Para tais estudos foi utilizado um modelo de camundongo que desenvolve fibrose hepática crônica (induzida por CCl_4 por um período de 8 semanas) propiciando a ativação de HSCs. Os conjugados foram previamente testados em células e mostraram-se eficazes, comprovando que o procedimento

de síntese para endereçamento ao receptor-alvo não afetou a atividade biológica da IL-10. *In vivo*, com o endereçamento da IL-10 para as HSCs em nosso modelo animal os efeitos foram, para nossa surpresa, o contrário do esperado, ou seja, ao invés de melhorar a fibrose hepática, o conjugado mostrou-se pró-fibrótico. Paralelamente a isso, encontramos mudanças em marcadores de macrófagos nos fígados com fibrose de animais tratados com o conjugado quando comparados com os tratados com IL-10 não modificada ou com os de camundongo que não receberam tratamento algum. O perfil dos macrófagos nos fígados dos animais que receberam PPB-PEG-IL-10 era composto, em sua maioria, por um subtipo relacionado ao processo de cicatrização, que estimula o depósito de colágeno em área de injúria tecidual. Como embora houvéssemos enviado a Interleucina-10 para as HSCs, mas observamos efeitos em macrófagos (que não apresentam receptor PDGF) prosseguimos e aprofundamos nossa pesquisa na população de macrófagos hepáticos e nas interações destes com as HSCs (resultados descritos no capítulo 5). Esses resultados mostraram que nos fígados dos camundongos tratados com PPB-PEG-IL-10 houve um aumento significativo na proteína quimioatraente de monócitos/macrófagos-1 (CCL2/MCP-1), assim como em seu receptor (CCR2). Na literatura foram publicados estudos que mostram que as céluas estreladas hepáticas são capazes de estimular a secreção de CCL2 por macrófagos e nossos dados sugerem que o subtipo de macrófago presente nos fígados dos animais tratados com o conjugado é do subtipo M2c. Esse subtipo de macrófago produz o fator transformador de crescimento beta (TGF-β), que é um fator pró-fibrótico. Com base nos resultados obtidos e descritos nos capítulos 4 e 5, nossa hipótese é a de que a Interleucina-10 desempenha um papel importante em fibroblastos: esta citocina estimula a produção de CCL2 por HSCs que atraem macrófagos (positivos para CCR2); esses macrófagos, por sua vez, estimulam o processo de cicatrização tecidual e como resultado há um aumento da deposição de colágenos. Assim sendo, enquanto a Interleucina-10 não modificada tem um efeito anti-fibrótico, que pode até ser amplificado caso a sua meia-vida no plasma seja aumentada, a sua forma conjugada, com edereçamento específico para HSCs tem um efeito pró-fibrótico.

Conclui-se no **capítulo 6**, o novo conhecimento gerado por nossos estudos a respeito do papel da Interleucina-10 na fibrose hepática. A administração intra-venosa de Interleucina-10 afeta o processo fibrótico de várias maneiras, levando a resultados anti-fibróticos através de um efeito anti-inflamatório e a efeitos pró-fibróticos quando endereçada às HSCs, através da atração indireta de macrófagos relacionados ao reparo tecidual. Os dados da literatura acerca da IL-10 são contraditórios e nossos dados oferecem explicações para algumas dessas contradições. O efeito da IL-10 depende do tipo celular predominante no fígado doente, da composição entre células inflamatórias, macrófagos e células estreladas. Essa composição pode variar consideravelmente desde a fase inicial da doença (muita atividade inflamatória) até

a fase tardia (com muita atividade fibrótica e aumento de HSCs ativadas). Nossos resultados podem também explicar os resultados contraditórios em diferentes modelos animais relatados na literatura, pois estes também apresentam populações de células hepáticas em diferentes proporções de células inflamatórias, macrófagos e células estreladas. Nossos dados também sugerem um papel fundamental dos macrófagos M2c durante o processo fibrótico. Uma possibilidade de tratamento da fibrose hepática seria através do endereçamento de substâncias terapêuticas especificamente para os macrófagos M2c, para inibir suas atividades pró-fibroticas, mas isso requer uma investigação mais aprofundada. Nossos estudos também mostram que o endereçamento celular específico de citocinas é possível e isso fornece uma base sólida para novas oportunidades terapêuticas para combater essa patologia crônica que é a fibrose hepática.



Acknowledgements

"A gente não faz amigos, reconhece-os."
Vinícius de Moraes

Some years ago I've decided to leave Rio de Janeiro, known as the "wonderful city" (cidade maravilhosa) to start a new adventure in Groningen, The Netherlands. In the beginning it was really hard to adapt to a complete new situation and to get used to so many different things at the same time. As time went by, this charming small city in the far-north of The Netherlands conquered my heart and became my home.

It is well-known that immigration has a high impact in a person's life; It's a difficult decision and the feeling is comparable to grieving. Well, I see it as an opportunity to shape a totally new life and I can assure you that there are no regrets!

The people who gave me support, opportunities and happiness are the main reason which defined my immigration experience as a good and exciting one. And now is the time to acknowledge all these people that directly or indirectly contributed to my "new life" and PhD.

First of all I would like to thank my supervisor, Prof. dr. Klaas Poelstra. **Klaas**, I admire your competence, creativity and fantastic solutions. You are that sort of person that nobody can forget. Your enthusiasm for science and always positive posture are an example that must be followed. Thanks for receiving me in your lab and for all these years of guidance! Oh, and also thanks for your infinite patience ☺

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At the last months of my contract at RUG, I was concerned about getting another job. I was eager to have a teaching position again, besides doing research. At **Hanze**

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In the Dutch language the verbs "to teach" and "to learn" are the same (leren) and nothing could be truer. I would like to let **my students** from HIT know that I've learned a lot with them.

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It would not have been possible to be here if it was not for my Brazilian family. I will always be grateful to my mom, dad, aunt, grandmas (*in memoriam*), sister + brother in law + baby Livia, brother and cousins.

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Adriana